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Loop-mediated isothermal amplification (LAMP) for the diagnosis of human sleeping sickness: towards a point-of-care diagnostic test.

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Declaration

I declare that the research described within this thesis is my own work and that this thesis is my own composition

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Abstract

Acute and chronic sleeping sickness are fatal neglected tropical diseases caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* respectively (members of the sub-genus *Trypanozoon*). Accurate diagnostics are needed to guide treatment since the symptoms of disease are non-specific and the drugs that are used for treatment are too toxic to be administered to unconfirmed cases. Tests need to be simple enough to confirm clinical diagnosis of sleeping sickness in poorly-resourced, peripheral health centres and for use as epidemiological tools to detect *T. b. rhodesiense* in the zoonotic reservoirs of infection.

This study focuses upon LAMP (loop-mediated isothermal amplification) as a novel diagnostic for sleeping sickness that may serve to bridge the gap between the need for sensitive, specific molecular diagnostics on the one hand and 'field-friendly' diagnostics on the other.

Here, two previously published LAMP assays for *Trypanozoon*s were compared to classic PCR based methods for the diagnosis of *Trypanozoon* infection status in 428 cattle blood samples. The results did not support the use of LAMP as an improved system for surveillance of *T. b. rhodesiense* in the zoonotic cattle reservoir.

T. b. rhodesiense and *T. b. gambiense* subspecies specific LAMP assays were evaluated against traditional reference subspecies specific PCR tests, using DNA purified from 86 cryopreserved trypanosome isolates. Novel LAMP assays for these subspecies were also designed and evaluated. Both the published and novel assays for *T. b. rhodesiense* (targeting different regions of the *SRA* gene) were sensitive, specific and reliable when applied to purified DNAs, but were less consistent on field samples. The novel *T. b. gambiense* LAMP (targeting *TgsGP*) was sensitive and specific but this was not the case for the published LAMP assay (targeting the 5.8S rRNA gene). However reliability may be less than optimal for LAMP *TgsGP*.

Finally, simple endpoint readout methods for LAMP were evaluated. The colour change reagent hydroxynaphthol blue was identified as the best currently available method taking cost, ease of use and reliability into consideration.

In 2009 the number of reported sleeping sickness cases fell below 10,000 for the first time in 50 years. Improved LAMP diagnostics could facilitate the diagnosis of sleeping sickness and support the continued fight against this neglected, but deadly disease.

1 Chapter 1. Introduction

1.1 Diagnostics for Global Health

'Access to appropriate diagnostic tools is an essential component in the evaluation and improvement of global health' [1].

This statement, made in the Nature supplement 'Determining the Global Health Impact of Improved Diagnostic Technologies for the Developing World' in 2006, highlights several of the key issues that remain pertinent to Global Health today.

Firstly, effective diagnosis and the availability of diagnostic tools are an essential component of global healthcare (despite their often low-key status next to drugs and vaccines [1, 2]). They are crucial because they enable the current health status (of individuals and populations) to be evaluated in order that rational strategies for improvement can be made.

Secondly, diagnostics must be appropriate for purpose and setting. They must meet specific user requirements as well as performance standards. It is not often possible or appropriate to develop a single tool with multiple diagnostic objectives. Therefore the aims and application of any proposed diagnostic tool should be clearly defined [1].

Thirdly, diagnostics must be accessible. To a large extent accessibility is determined by the setting for which diagnostic tools are designed. Where end user requirements are misunderstood diagnostic tools will never be accessible to those who need them [1].

1.1.1 The roles of diagnostic tests for infectious diseases

Diagnostic tests and techniques are designed and applied for different purposes. A single diagnostic might serve one or more diagnostic objectives which may include: (i) patient management, including treatment follow up; (ii) screening for asymptomatic diseases; (iii) surveillance (including verification of elimination); (iv) monitoring the effects of public health interventions; (iv) outbreak investigations and epidemiological studies; (v) detection of infections with drug resistance markers [1, 3].

Throughout this thesis the term 'diagnostic' is used in its broadest sense as any tool that reduces uncertainty about the state of infection or disease. In addition to diagnostic tests used for case confirmation among symptomatic individuals, it is also used to describe screening tests used for case detection in asymptomatic cases [4, 5].

1.1.2 Diagnostics in the developing world

Recently 'modified molecular diagnostics for affordable, simple diagnosis of infectious diseases' was ranked as the most promising biotechnology for improving health in developing countries. In a study which sought consensus opinion among 28 scientific experts (with expertise in public health problems of developing countries), molecular diagnostic tools were collectively ranked above biotechnologies for both vaccine development and drug discovery [6].

However, there are many challenges in providing appropriate diagnostics in the developing world.

1.1.2.1 Barriers to the development and uptake of appropriate diagnostics in the developing world

Diagnostic tools have been typically developed by industrialised countries for use and application in those industrialised country settings. As a consequence, they may often be ill suited for use in the developing world. They may be too expensive or technologically demanding for resource-poor settings. They may need to be executed in centralised facilities and require considerable training of the end users, most of which are not available in developing countries with limited health care budgets and facilities [6, 7]. Even where centralised reference laboratories are not a necessity, a good quality laboratory service is still needed, which includes technical competence, access to the proper reagents, an understanding of quality control and proper interpretation of diagnostic test results by healthcare providers as well as a timely system for the communication of test results between technicians and the healthcare providers [8]. At present there often exists a cycle of neglect, whereby poor laboratory services generate untrustworthy results, leading to the devaluation of the laboratory services by clinicians, undermining opportunities for improvement [8]. In sub-Saharan Africa the variable and often poor quality of laboratory services remains a barrier to effective healthcare [9, 10]. There is a lack of investment in diagnostic services development; for example, in a Malawian district hospital only 6 % of health expenditure was on diagnosis [11] . Further, diagnostics might not have been properly and rigorously validated in developing world settings [12, 13]. The ethical and strategic importance of developing country involvement in the diagnostic evaluation process has been noted elsewhere [2]. Apart from tests used for blood banking, regulatory standards are often lacking for diagnostic tests, especially those uncommon in industrialised countries [3, 14]. Even where appropriate and effective tests exist, advocacy might be insufficient to generate the necessary levels of uptake [15] and there may be inadequate quality control of tests once they are implemented [16, 17].

Historically there have been few incentives and little impetus to address this imbalance. This could be attributed to a private sector perception that diagnostics

generate poor investment returns, lack of information about the requirements for high impact diagnostics in the developing world, and lack of information about the potential health benefits appropriate diagnostics could bring [7].

Interest in diagnostics for the developing world has begun to grow. Over the past decade funding has improved [8] and several large-scale initiatives have been launched to develop novel diagnostic technologies and improve access to novel and existing diagnostics. These new initiatives include the Foundation for Innovative New Diagnostics (FIND), the Global Health Diagnostics Forum and the African Network for Drugs and Diagnostics Innovations (ANDI).

1.1.2.2 The Foundation for Innovative New Diagnostics (FIND)

FIND was established as a product development and implementation partnership seeking novel and improved solutions for the diagnosis of sleeping sickness, malaria and tuberculosis. FIND is a non–profit Swiss based foundation which was launched on 22nd May 2003 at the World Health Assembly (see www.finddiagnostics.org) as a spin-out from the World Health Organisation (WHO) and TDR (Special Programme for Research and Training in Tropical Diseases, executed by the WHO and co-sponsored by the United Nations Children's Fund (UNICEF), the United Nations Development Programme (UNDP), the World Bank and the WHO). Their work on diagnostics for sleeping sickness is included in Section 1.3.

FIND is not the first public private, product development partnership with a remit which includes diagnostic technologies. The Program for Appropriate Technology in Health (PATH) was established in 1980, from the Program for the Introduction and Adaptation of Contraceptive Technology (PIACT). More recently, the Center for Point-of-Care Diagnostics for Global Health was established as a collaboration of PATH and the University of Washington. The center focuses on (i) clinical needs assessment for technology developers; (ii) supporting exploratory projects; (iii)

clinical testing of prototype point-of-care diagnostics; and (iv) training on the clinical realities of designing point-of-care products for low-resource settings. It is funded by the National Institutes of Health (NIH), National Institute of Biomedical Imaging and BioEngineering (NIBIB). See www.path.org.

Another, notable example is the Infectious Diseases Research Institute (IDRI). Since it was founded in 1993, IDRI has developed diagnostic tests for Chagas disease, tuberculosis and leishmaniasis. See www.idri.org.

1.1.2.3 Global Health Diagnostics Forum

The Global Health Diagnostics Forum was convened in 2004, by the Bill and Melinda Gates Foundation (www.gatesfoundation.org) and the RAND Corporation (www.rand.org). The forum includes expertise in specific diseases, disease modelling, the introduction and adoption of diagnostic technologies, and includes representatives from the diagnostics industry and technology development sector. The forum has developed a mathematical modelling approach to quantify the potential health impact of novel diagnostics in developing countries, and to define the performance characteristics and user requirements necessary for high impact diagnostics [18]. A summary of their findings is given in Appendix 1. For example, they have estimated that a malaria diagnostic with no infrastructural requirements, 90 % sensitivity and 90 % specificity would avert 300,000 child deaths and 450 million unnecessary treatments annually.

1.1.2.4 African Network for Drugs and Diagnostics Innovations (ANDI)

Potential benefits from the development of novel diagnostics are not solely restricted to health *per se*. The African Network for Drugs and Diagnostics Innovations (ANDI), launched in October 2008, includes representation from 21 countries committed to coordinating their efforts to encourage research and innovation for Africa-centric health problems. Like FIND, ANDI was initiated by WHO/TDR. It is supported by WHO/TDR, the African Development Bank and the European Union, as well as governments and pharmaceutical companies. ANDI suggest that Africa does have the capacity to develop new drugs and diagnostics but have identified a widespread lack of translational mechanisms to take findings from the laboratory to commercialisation on the continent [19]. They also suggest that 'a creative public-health-centered environment for intellectual property management and licensing structures could advance health and support innovation in Africa' [19] such that intellectual property rights could be used to as a driver of local innovation for local health problems. Such a strategy could bring economic development benefits, in addition to the direct health benefits from a particular innovation. In addition the WHO convened an Inter-governmental Working Group on Innovation, Intellectual Property and Public Health, which has developed a Global Strategy and Plan of Action to lower the barriers for public health innovation by creative intellectual property management strategies.

1.1.2.5 The future

In order to overcome the challenges outlined above there is a need for: advocacy; continued technological advancement towards ideal point-of-care tests; the development of harmonized regulatory standards; improved evaluation and uptake of diagnostic tests; capacity building, quality assurance and education of healthcare providers [8].

Although funding has improved, advocacy for diagnostic leadership in developing countries is needed.

In 2003 WHO/TDR coined the acronym 'ASSURED' to summarise the characteristics of an ideal point-of-care test. These are: Affordable; Sensitive; Specific; User-friendly (simple to perform in a few steps with little training); Robust and rapid (can be stored at room temperature and results available in less than 30 minutes); Equipment free (or minimal) and Deliverable to the point of need. Technological advances are being made which are likely to generate more tests that fulfil all these criteria over the nest few years. In this thesis the focus is on LAMP (loop-mediated isothermal amplification) as a new technology that might provide an ASSURED test for sleeping sickness.

Regulatory standards would prevent the market being flooded with cheap, low quality tests. However, financing a product through the regulatory process is costly, therefore, harmonized international or regional regulatory standards would encourage the diagnostic industry to register their products in many countries. In addition an international federation of diagnostics manufacturers (similar to the International Federation of Pharmaceutical Manufacturers and Associations) which could set quality control standards, as well as working with governments to combat counterfeiting, would represent a leap forward.

Technological advancement and regulation alone will not be enough. In addition significant capacity building and education are required to achieve laboratory services for diagnostic testing. In particular rigorous quality control systems will need to be implemented and facilitated. If this is achieved, clinicians may start to put their trust in test results, breaking the cycle of neglect described above.

1.2 Sleeping Sickness

Sleeping sickness or human African trypanosomiasis (HAT) comprises two forms of a neglected tropical disease whose reported incidence (9,877 new cases in 2009 [20]) disguises a high disease burden, not least due to severe under reporting. Indeed, a recent study has highlighted the need for localised estimates of disease burden which account for under reporting [21]. Chronic sleeping sickness, caused by infection with *T. b. gambiense* parasites and acute form sleeping sickness caused by *T. b. rhodesiense* are both fatal if un-treated and yet diagnosis and treatment remain highly problematic. Although there are many different diagnostic approaches (Section 1.3) the much sought after sensitive, specific and low-resource tool remains elusive. Drugs for sleeping sickness are either highly toxic, complex to administer or both. Drug resistance is of increasing concern and treatment costs are extremely high. For example, individual treatment costs for *T. b. rhodesiense* have been estimated to be almost eight times higher than the per capita government health expenditure in Uganda, where the total costs of treating a Rhodesian sleeping sickness case has been estimated at US $147 in comparison to US $19 of government funding available for per capita healthcare in 2008 [22]. In the Democratic Republic of Congo Gambiense sleeping sickness costs have been estimated to average an equivalent to five months income for an affected household. Similar proportions of income are required to cover the direct and indirect costs of Rhodesian sleeping sickness for families living on less than US $1 day^{-1} in Tanzania [21].

1.2.1 Parasites, host and vectors

The causative agents of human sleeping sickness are parasites of the genus *Trypanosoma*, subgenus *Trypanozoon*. The *Trypanozoon*s include *Trypanosoma brucei sensu lato* (s.l.), *Trypanosoma equiperdum* and *Trypanosoma evansi*, of which only *T. brucei* s.l. subspecies cause human sleeping sickness (although there has been a reported case of *T. evansi* infection in an immunocompromised individual [23]).

T. brucei s.l includes three (at least) subspecies of which two cause human sleeping sickness: *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. These parasites are morphologically indistinguishable from each other and also from the third, non-human-infective member of the *Trypanosoma brucei* s.l. species complex, *Trypanosoma brucei brucei*. At the molecular level, *T. b. rhodesiense* can be discriminated by the *SRA* gene [24]. Similarly, *T. b. gambiense* can be distinguished by the presence of the *T. b. gambiense* specific *TgsGP* gene [25]. The discovery of these subspecies specific diagnostic marker genes is described in more detail in Section 1.5. *T. equiperdum* and *T. evansi* are now consided to be strains of *T. brucei* rather than separate species [26], while previously this was uncertain [27].

Despite their morphological homogeneity *T. b. brucei*, *T. b gambiense* and *T. b. rhodesiense* differ in both host range and disease profile. *T. b. brucei* infects a range of domestic and wild mammalian hosts, with varying degrees of pathogenicity. For example, while indigenous African cattle are generally unaffected, the parasite is pathogenic in exotic cattle breeds, as well as horses and dogs [22]. *T. b. brucei* is not infective to humans; who have innate resistance conferred by apolipoprotein L-1 (apoL1) and haptoglobin related protein (Hpr), present in normal human serum [28, 29]. The human infective parasite *T. b. rhodesiense* has long been known to exist as a zoonotic disease, infecting both wildlife and domestic livestock [30, 31]. Recently the critical role of these reservoirs has become increasingly apparent; rapid spread of acute sleeping sickness in Uganda has been attributed to the movement of livestock (up to 18 % of which were shown to be infected with *T. b. rhodesiense* [32, 33]). In contrast, the role of an animal reservoir for *T. b. gambiense* is less certain; Gambian sleeping sickness is thus considered to be principally anthroponotic [34], although domestic pigs are known reservoirs of infection and may play a role in disease transmission [35]. Table 1.1 summarises the key characteristics of *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*.

Table 1.1. Similarities and differences between the *T. brucei* s.l. subspecies

Parasite	Principal hosts	Clinical profile	Geographical profile
T. b. brucei	Wildlife, domestic livestock	Clinically insignificant	Pan-African
T. b. gambiense	Humans	Chronic disease	West and Central Africa
T. b. rhodesiense	Wildlife, domestic livestock and humans	Acute disease in humans, clinically insignificant in the zoonotic reservoir	East Africa

All *T. brucei* s.l. are vector borne parasites that are cyclically transmitted between mammalian hosts by both male and female tsetse flies of the genus *Glossina* [36].

Trypanosoma evansi is transmitted mechanically by haematophagous insects such as tabanid flies and causes Surra, a disease that affects wild and domestic animals [23, 37]. *Trypanosoma equiperdum* is traditionally considered to be the causative agent of the equine disease Dourine, transmitted by direct, e.g. sexual, contact between animals. However, the classification of *T. equiperdum* as a distinct species remains controversial; it may be a mis-classified *T. evansi* or another member of the *T. brucei* subspecies [38]. Some authors have gone further to suggest that both *T. evansi* and *T. equiperdum* are both subspecies of *T. brucei* s.l. lacking part or all of their kinetoplast DNA [26].

In addition there are several animal infective trypanosomes, which cause 'sleeping sickness' or Nagana in animals and which fall into two other subgenera. The subgenus *Duttonella* includes *Trypanosoma vivax*, while the subgenus *Nannomonas* includes *Trypanosoma congolense*, *Trypanosoma simiae* and *Trypanosoma godfreyi*.

Conventionally, it is believed that only *T. b. rhodesiense* and *T. b. gambiense* are human infective. However, exceptional atypical infections with *T. b. brucei* [39], *T. evansi* [23] and *T. congolense* [40] have been reported.

1.2.2 Lifecycle

A susceptible tsetse fly becomes infected with bloodstream form trypanosomes when it feeds upon an infected host. This establishes a dividing population of procyclic form trypanosomes within the tsetse midgut. In the case of *T. brucei* s.l. these then migrate to the tsetse salivary glands and mature to the metacyclic, mammalian infective, stage. The transmission cycle is completed when a second mammalian host is infected during a subsequent blood meal. The tsetse is not very hospitable to the trypanosome and has evolved several defence mechanisms against the invading parasite, indeed most flies are refractory to infection [41]. Furthermore, human-infective trypanosomes are less able to mature within the fly than their human serum sensitive counterparts [42].

Metacyclic stage trypanosomes are injected into the mammalian host via the tsetse proboscis and rapidly transform to bloodstream stages. Infection becomes systemic and the trypanosomes multiply by binary fission in lymph and blood, but the mammalian host mounts a lytic antibody response against the variable surface glycoprotein (VSG) outer parasite layer, leading to a reduction in the number of parasites in the blood. However, by stochastic antigen switching of the VSGs the parasite changes its coat [43], so that a subpopulation of parasites avoids destruction. This occurs repeatedly, generating waves of parasitaemia, each of a different variable

antigenic type (VAT). Therefore the infection is maintained, ready for transmission during the next tsetse blood meal. During the later stages of the disease the parasites cross the blood brain barrier and also multiply in the cerebrospinal fluid.

1.2.3 Clinical features

The clinical course of human sleeping sickness may be divided into two distinct stages, which may or may not be preceded by chancre development at the site of parasite inoculation. The early stage of the disease corresponds to the infection of the host haemolymphatic system, and is characterised by non-specific clinical signs, particularly fever and lymphadenopathy. The late stage of the disease develops upon trypanosome invasion of the central nervous system via the blood brain barrier, leading to meningitis and encephalitis. Clinical manifestations include headaches, backache and neck stiffness, behavioural and psychological changes, profound disturbance of the sleep-wake cycle, coma and death.

These stages are common to both Rhodesian and Gambian sleeping sickness; though the rate of disease progression differs markedly between the two. Gambian sleeping sickness is a chronic disease with early stage symptoms appearing weeks or months after infection and if untreated death may not occur for many years [44]. However, recently this traditional view has been challenged, and evidence for spontaneous cure as well as chronic carriage has been reviewed by Checchi *et al.* [45]). By contrast Rhodesian sleeping sickness follows a more acute clinical progression in which the two stages of the disease are less distinct, and where if untreated, 80% of deaths normally occur within six months of infection [44, 46]. However, across the geographic range of *T. b. rhodesiense* there is a spectrum of disease severity. In the northern part of the range, the disease is classically acute, such as in Uganda where death occurs within six months of the onset of illness in more than 80 % of reported cases [46]. In the southern part of the range, the disease has long been considered to be more chronic and human 'carriers' have been reported [47]. The observed clinical differences correlate with genetic differences reported between parasite strains

isolated from the northern and southern parts of the distribution range; observed by isoenzyme [48, 49], restriction fragment length polymorphism [50] and minisatellite [51] analyses. The north south divide is also characterised by small differences in the *SRA* gene [52, 53] (discussed in more detail below) as well as differences in host inflammatory cytokine response profiles [54].

1.2.4 Epidemiology

Sleeping sickness is a disease affecting poor rural communities who are exposed to tsetse as part of their daily activities such as agriculture, fishing, animal husbandry and hunting. However, epidemiologically Gambian and Rhodesian sleeping sickness are dissimilar and, at present, spatially allopatric. Chronic sleeping sickness, caused by *T. b. gambiense*, is an anthroponotic disease restricted to West and Central Africa, whereas *T. b. rhodesiense* in East Africa is zoonotic, with an acute clinical progression in humans.

Both forms are characterized by discreet foci of disease which persist over time, expanding and contracting during epidemic and inter-epidemic periods and which are strictly restricted to tsetse inhabited regions of sub-Saharan Africa [22]. Yet even within these areas sleeping sickness does not present with a uniform distribution and the abundance of tsetse flies is not the only limiting factor for disease. Animal reservoirs of disease are an important determinant of disease distribution, and population displacement, war and poverty all seem to contribute to exacerbate transmission. Recently Batchelor *et al.* [55] observed an important correlation between the proximity to livestock markets and the prevalence of Rhodesiense sleeping sickness in newly affected areas of Uganda, as well as noting correlations with measures of green vegetation, land surface temperature and the proximity to health facilities. Thus social, environmental and climatic factors all play a role: the epidemiology of sleeping sickness is complex and many features remain unexplained.

Given the focal nature of these diseases focal estimates of disease burden are required to truly represent the impact of the disease, and inform control [56]. This was achieved for a highly endemic region of Tanzania in a recent study by Matemba *et al.* [21].

More than 250 endemic foci of disease have been recorded in 36 countries across sub-Saharan Africa [57]. Despite the current geographic separation of the two human infective parasites, concerns have recently been raised about the potential merging of foci in Uganda where the two forms are separated by a buffer of only 150 km [32]. This would have serious implications for disease control in terms of surveillance, diagnosis and treatment [32, 55].

1.2.5 Control

Past experience indicates that control is feasible: towards the end of the colonial era, by the late 1960s, sleeping sickness was considered all but conquered. Mobile surveillance for early case detection and treatment formed the core of the control initiatives. Sadly, the disease showed a dramatic re-emergence during early independence as control programmes became a victim of political instability and their own success [58, 59]. Renewed efforts have seen case numbers falling once again [59, 60]. However, in contrast to successful historical models of sleeping sickness management, current control strategies are almost entirely reactive, only coming into play in response to major epidemics [61].

According to Molyneux *et al.* [62], those afflicted by sleeping sickness have been failed by the public health and scientific communities alike. Public health policy makers and practitioners have failed to sustain simple, proven methods of control. As for the scientific community, the priorities for research towards improved control have changed very little over the last half century (cheap, point-of-care diagnostics

and effective, non toxic drugs for late stage disease) but progress towards these targets has been impeded by the diversion of research efforts into scientifically impressive investigations into trypanosome and tsetse biology that are remote from the needs of the patient. Molyneux *et al.* summarise the problems and future requirements in the following statement, "*Today we are able to undertake the most elaborate scientific experimentation on tsetse and trypanosomes, yet we are barely able to manage sleeping sickness during the comfort afforded by the present inter-epidemic period. The huge rise in philanthrocapitalist investments that has been welcome in the past decade now needs to translate into practical solutions for rural peoples to manage this devastating disease [17]. Investments that we have seen in genetics and genomics may reap rewards in years to come, but in the meantime, funds must be provided to sustain effective, if unsexy, control strategies.*"

Having noted that control is realistic, control strategies are now described in more detail.

1.2.5.1 Differences in control strategies for Gambian and Rhodesian sleeping sickness

Control strategies for Gambian and Rhodesian sleeping sickness differ. Control of Gambian sleeping sickness relies on early case detection and treatment to reduce the anthroponotic parasite reservoir. Active screening is necessitated by the long asymptomatic preclinical stage during which infected individuals are likely to transmit disease without seeking treatment. Control of Rhodesian sleeping sickness requires additional strategies to reduce the parasite population in its zoonotic reservoir. Active case finding is less important; the acute nature of the disease mean that cases are more likely to self-present at health centres [34]. The requirement for and effects of targeting the livestock reservoir of *T. b. rhodesiense* in south east Uganda have been quantified [61], and are discussed in more details in Section 1.2.5.3 (Control in the animal host) below.

1.2.5.2 Control in the human host

Case detection and subsequent curative treatment in the human host prevents onward transmission. However, both case detection and treatment remain difficult and inadequate. Quantification of the level of under detection of *T. b. rhodesiense* sleeping sickness during an epidemic in Uganda [63] found that only 20 % of sleeping sickness cases present with early stage disease and that for every fatality another twelve go undetected. Furthermore, 85 % of undetected deaths remain undiagnosed despite having entered the health system. Therefore, diagnostic delays can be attributed to the service providers as well as the care seekers. These delays worsen the prognosis at treatment and increase the likelihood of onward transmission [64]. Specific diagnostics and their limitations are described below (Section 1.3).

Current chemotherapy for human sleeping sickness also remains unsatisfactory. Problems include a limited and ancient repertoire of effective drugs, high frequency of severe adverse events, complex drug administration typically requiring hospitalization and drug resistance [65]. At least for now the constant threat to sustainable drug production (due to low profitability) has been overcome, since the pharmaceutical industry agreed to provide drugs for free [62].

Treatment depends upon the infecting parasite and the stage of disease progression. There are four registered drugs to choose from. Table 1.2 outlines the treatment options.

Table 1.2. Chemotherapy of human African trypanosomiasis [66, 67].

Drug	**Adverse events**	**Administration** (Preferred method)	**Effective against** (Drug of choice for)	
			Infecting parasite	**Stage**
Suramin	Generally well tolerated.	Intravenous injection over 15-30 days	*T. b. rhodesiense, T. b. gambiense*	Early
Pentamidine	Generally well tolerated.	Intramuscular injection or intravenous infusion over 7-10 days.	*T. b. gambiense*	Early
Melarsoprol	Drug induced encephalopathy in 5-10% of patients (70% fatal). Exfoliative dermatitis in <1%	Intravenous injection over 7-10 days	*T. b. rhodesiense, T. b. gambiense*	Early, late
DFMO	Frequent. Bone marrow toxicity, gastrointestinal and neurological symptoms.	Orally every 6 hours for 14 days. Or intravenous infusion.	*T. b. gambiense*	Early, late
Nifurtimox – Eflornithine Combination therapy	Reduced side effects compared to DFMO alone.	Oral nirfutimox daily for 10 days, 12 hourly intravenous infusions DFMO 7 days.	*T. b. gambiense*	Late

The drugs for human sleeping sickness have been in use for several decades and new, less toxic drugs are urgently needed. Suramin, which was introduced in 1920, is

effective against first stage Gambian and Rhodesian sleeping sickness. However pentamidine, introduced in 1940, is preferred for the Gambian form because administration is more simple and rapid. Melarsoprol, introduced in 1949, is an organo-arsenical compound. In addition to the severe adverse events outlined above, it also gives rise to more minor skin reactions, pyrexia, headache and general malaise in nearly all patients. D,L-α-Diflouromethylornithine (DFMO) is a viable alternative for second stage Gambian sleeping sickness but it is often prohibitively expensive, and being only trypanostatic, is not effective in patients with compromised immune systems, for example, HIV positive individuals. Drug bioavailability is much better when administered by intravenous infusion. Melarsoprol and DFMO are not used to treat early stage disease because of their toxicity and expense respectively.

Diminazene aceturate has been developed and registered for use against animal trypanosomes but has not been registered for human treatment. However, it has been applied intramuscularly against the first stage of both disease forms and appears to be effective. It is cheap and readily available but its toxicology in humans is unknown [67].

Recently a new treatment regimen has been established. In 2009, the WHO added Nifurtimox- Eflornithine Combination Therapy (NECT) to their model list of essential medicines and began distribution, following a randomized trial comparing standard eflornithine to NECT [68]. WHO has signed an agreement with Bayer Schering Pharma for the free supply of 400, 000 doses of Nifurtimox, per year, for five years, adding to a pre-existing donation of eflornithine by Sanofi Aventis. Previously nifurtimox was registered for use against Chagas disease (American trypanosomiasis) but not for human African trypanosomiasis (HAT). Compared to eflornithine treatment costs are reduced by half and hospitalization is reduced from 14 to ten days. Side effects are reduced compared to both melarsoprol and eflornithine, and it is at least as efficacious as eflornithine monotherapy [69]. Although this is a real step forward in improving treatment options many problems

remain, including transportation of the drug to remote health posts, intravenous administration and the specialized medical care needed to monitor for side effects as well as follow up for relapsing cases [62]. Drug resistance is also a concern. The nitro drugs, including nifurtimox and fexinidazole (a clinical trial candidate) have been shown to rapidly acquire reciprocal cross resistance, without compromising parasite virulence. Neither should therefore be considered as a stand alone treatment [70].

The discovery of a potent and selective *N*-myristoyltransferase inhibitor, currently designated DDD85646, that could cure experimental infections in mice with an oral dose administered over four days may well represent an opportunity for the development of a safe oral treatment against this neglected disease. Trypanosomes appear to be hypersensitive to inhibition of this enzyme. Further work is now underway to improve the CNS penetration and selectivity this model *T. brucei N*-myristoyltransferase inhibitor [71].

1.2.5.3 Control in the animal host

Mathematical modelling has clearly demonstrated that control of *T. b. rhodesiense* in the animal reservoir, in addition to early case detection and treatment in humans, is critical for the control of Rhodesian sleeping sickness [61]. Specifically, Welburn *et al.* [61] developed Rogers seminal mathematical model [72], which described the transmission of *Trypanosoma brucei* between two host populations by a single vector, to incorporate the effects of medical and veterinary interventions [34], and then parameterised the model using field-based estimates from south east Uganda [61]. Welburn *et al.* calculated that cattle account for 92 % of the total transmission potential for *T. b. rhodesiense*. Further, they calculated that mass chemoprophylactic treatment which reaches 86 % of cattle can prevent outbreaks of Rhodesian sleeping sickness [61]. (This estimate is relatively insensitive to the percentage of humans receiving treatment). In short, in the absence of the cattle reservoir the basic

reproductive number (R0) (which is the number of secondary infections arising from one infectious individual entering a fully susceptible host population) of *T. b. rhodesiense* falls below zero and transmission cannot be maintained.

Control of the human-infective parasite in the domestic livestock reservoir relies on three trypanocidal drugs, all of which have been in use for over 40 years. Isometamidium chloride has prophylactic as well as therapeutic properties, homidium salts have limited prophylactic use while diminazene aceturate is solely therapeutic. Drug resistance is a serious problem for animal trypanosomiases [73] and cross resistance is seen with drugs used to treat human disease [74]. Novel drug development is commercially unattractive. Therefore, these three established drugs should be used carefully and the prevalence and spread of drug resistance ought to be monitored. New methods to rapidly assess the drug susceptibility status of circulating trypanosomes are needed [74].

In Uganda there is a well-designed national policy for the trypanosomiasis control, including treatment of cattle at their point of origin or before sale. However it is clear that this policy has not been effectively implemented [75].

1.2.5.4 Control of the tsetse vector

Controlling the tsetse population is a longstanding and well-established approach for trypansomiasis control. It has been well described elsewhere [58, 76]. Here it is sufficient to note that despite limited but notable success stories, such as the eradication of tsetse from Unjuga, Zanzibar, tsetse control is frequently unsustainable. For example, political unrest in Uganda in the 1970s interrupted a successful ground-spraying programme. Similarly, tsetse traps and targets have been shown to successfully control tsetse populations as part of government funded programmes, however, once left to the community they usually suffer the fate of the common good, and are not maintained. But tsetse control may yet be sustainably

achieved. The Stamp Out Sleeping Sickness programme has demonstrated that restricted application of insecticide to cattle has human and animal health benefits and by spraying cattle the common good problem is overcome [62].

1.3 Diagnosis of sleeping sickness

WHO recommends that a positive diagnosis should be made when parasites can be detected in the lymph or blood by microscopy [77]. This is followed by staging of the disease to determine the appropriate course of therapy. Staging guidelines are described in Section 1.3.5.

The differences in clinical progression seen with *T. b. gambiense* and *T. b. rhodesiense* necessitate the use of different diagnostic protocols. Although definitive diagnosis for both diseases relies on parasitological detection in the blood, or lymph, the steps preceding parasitological confirmation differ. For *T. b. gambiense* active serological screening and/or clinical signs are used to identify suspect cases whereas *T. b. rhodesiense* suspect cases are identified by the presence of trypanosomes in blood or CNS and associated clinical features.

1.3.1 Clinical suspicion

Clinical diagnosis of *T. brucei* infection is not possible in humans or cattle. In humans sensitive and specific diagnosis of sleeping sickness is not possible using clinical features alone but these features can be used to indicate that parasitological diagnosis is necessary [78, 79]. Cattle infected with *T. brucei* s.l. rarely show overt clinical signs [80] and other techniques are required to demonstrate the presence of the human infective parasite.

1.3.2 Serological screening

Several different techniques have been developed to detect trypanosome specific humoral responses in blood, serum and CSF samples, the most important of which is the card agglutination test for trypanosomiasis (CATT) [81]. CATT is a rapid, simple agglutination assay for anti-*T. b. gambiense* antibodies, which uses an antigenic reagent based on *T. b. gambiense* Variable Antigen Type LiTat 1.3. The CATT test is used to screen for *T. b. gambiense* in most endemic areas. During field surveys, the subjects are first screened using freshly collected heparinized whole blood. This is followed by further tests on blood, plasma or serum dilutions for blood test positive individuals. Seropositivity does not necessarily indicate current infection since antibodies can persist for up to three years post cure [82]. Therefore, CATT cannot be used as an indication of cure after treatment [83].

CATT false negatives have been observed in parasitologically positive cases in Cameroon and north western Uganda [84, 85]. Some CATT false positives might arise where the LiTat 1.3 gene is absent in circulating strains of *T. b. gambiense* [84, 86]. However, the CATT false negative cases in Uganda were LiTat 1.3 positive by PCR suggesting that LiTat 1.3 might not always be expressed, or may be expressed at a later stage in infection [85]. CATT false negatives can also result from complement-mediated inhibition, particularly at lower concentrations of sample [87]. To overcome this problem an improved version of CATT was developed, in which EDTA (which has anti-complement properties) is added to the dilution buffer [88].

CATT false positives are also likely. CATT false positives can occur in patients with malaria and transient infection with non-human-infective trypanosomes [81]. In the Cameroonian Fontem focus patient serum from CATT positive individuals failed to lyse LiTat 1.3 VAT trypanosomes, strongly indicating that CATT positivity can arise from cross reactivity [84]. Finally, high numbers of aparasitaemic serological suspects are observed. This might reflect a lack of CATT specificity or failure of the

parasitological parasitological tests in the face of weak or fluctuating parasitaemia [89, 90]. Perhaps, even, the patient's immune response is effectively controlling the parasite [91]. Attempts to validate CATT against molecular PCR tests have generated more questions than answers [92].

Other limitations include: (i) the complexity of antigen production which requires the extraction of trypanosomes from infected rat blood [78]; (ii) the requirement for a team of well trained personnel, and the transportation of bulky equipment; (iii) CATT tests are not available in an individual format; and (iv) the onerous follow up associated with the high number of observed CATT positive, but aparasitaemic individuals.

Modifications of CATT have been made for use with blood samples stored on filter paper, for example the Testryp micromethod [93] and CATT-FP [94]. These techniques require much lower quantities of CATT reagent, and could facilitate serological surveillance in particularly remote areas.

More recently a new format of CATT has been developed and shown to be comparable to the existing CATT in subjects from a highly endemic area of the Democratic Republic of Congo. The new format (CATT-D10) is based on a thermostable lyophilisation medium and is produced in 10 unit vials. It might be more suitable for use in peripheral health care facilities, which may lack cold chain, and may not see sufficient suspect cases to justify opening a traditional 50 unit vial of CATT antigen [95].

The LATEX/*T. b. gambiense* has been developed as a field alternative to CATT [96], and uses a combination of three surface antigens (LiTat 1.3, 1.5 and 1.6) coupled with suspended latex beads. The procedure is similar to that for CATT. In several field studies it was more specific, but less sensitive than CATT [78].

Immunoflourescence assays, ELISA and immune trypanolysis methods for anti-trypanosome antibody detection are also useful where there are good laboratory facilities, such as for the remote analysis of samples collected during large scale epidemiological surveys, but are not practical for application in the field [78].

There is no equivalent to CATT for serological screening for *T. b. rhodesiense* [97].

1.3.3 Parasitological diagnosis

Microscopic parasite detection, upon examination of the lymph, blood or cerebrospinal fluid of the human patients, provides direct evidence for parasite infection. Cervical lymph node (CLN) palpitation ought to be performed on all CATT positive *T. b. gambiense* suspect cases. When the CLN are enlarged, lymph node aspiration for direct examination is mandatory. Detection of circulating *T. b. rhodesiense* parasites is relatively easy, however, the numbers of circulating *T. b. gambiense* can range from 10,000 ml^{-1} to less than 100 ml^{-1}, which falls below the threshold of even the most sensitive methods [97].

Most simply parasites can be viewed in wet blood films, whereby blood is spotted onto the slide underneath a coverslip and examined. Thick blood smears enhance detection sensitivity compared to a wet blood film. The blood droplet is smeared, air dried out of direct sunlight, and fixed with Giemsa or field stain, before viewing.

Several concentration techniques have been developed to improve the diagnostic sensitivity of parasitological diagnosis. Triple centrifugation of blood was described by Yorke in 1938 [98]. Centrifugation of the blood sample in a capillary tube concentrates the trypanosomes in the white blood cell layer, which can then be examined using a normal light microscope (haematocrit centrifugation technique – HCT) [99] or a dark-ground or phase contrast microscope (buffy coat technique) [100]. The quantitative buffy coat technique combines centrifugation and fluorescent

detection using acridine orange [101]. The mini anion exchange centrifugation technique (mAECT) uses miniature anion exchange columns for the separation of trypanosomes from red blood cells prior to centrifugation [102].

The most sensitive techniques for the detection of parasites in lymph and blood are direct examination and mAECT respectively and when these two techniques were used in parallel sensitivity was improved further [103]. Recently an improved model for mAECT was developed by FIND and their partners in which several test components were redesigned. Most notably, a new collector tube and viewing chamber have made microscopic examination simpler for both tests [104].

Finally, *in vitro* isolation has been developed for isolation of trypanosomes under field conditions [105] but requires examination over several weeks.

Traditional microscopic methods for diagnosis are rapid and can be performed in the field. However, it is not possible to discriminate *T. b. gambiense* and *T. b. rhodesiense* by microscopy, and microscopy is insensitive when compared to molecular methods [106]. Table 1.3 describes the detection sensitivities of some of these parasitological methods.

Table 1.3. Comparative detection sensitivities of various parasitological diagnostic techniques [79]

Technique	Sensitivity (trypanosomes/ml)
Wet blood film	10,000
Thick blood film	5000
Haematocrit centrifugation	500
Mini anion exchange centrifugation	100

1.3.4 Molecular diagnosis

PCR assays have been developed to detect and differentiate trypanosomes at the species and subspecies levels. For *T. brucei* s.l. PCR targeting a 177bp repeat region can detect a DNA concentration equivalent to what is present in a single trypanosome [107]. For *T. b. rhodesiense* PCR assays targeting the single copy *SRA* gene have been developed. Of particular note is a multiplex reaction, which includes an internal control, to verify the presence of sufficient DNA for single copy gene detection. This assay can discriminate *T. b. brucei* from *T. b. rhodesiense* for a single trypanosome in most cases [108]. PCR based identification of *T. b. gambiense* is also possible, using primers designed to amplify the single copy *T. b. gambiense* specific glycoprotein (*TgsGP*) gene. Single round and nested PCR assays have been developed. The best reported detection limit is 10 trypanosomes per ml^{-1} [32, 109]. The *SRA* and *TgsGP* genes are discussed in more detail in section 1.5.

Hence, molecular methods are significantly more sensitive than microscopy. In our laboratory PCR is applied routinely to blood samples collected and stored onto Whatman FTA cards. Whatman FTA cards contain a chemically treated fibre matrix that lyses cells, inactivates proteins and immobilises DNA, making them suitable for long term storage of blood samples. This approach enables accurate species-specific trypanosome identification for large-scale epidemiological surveillance at distant

field sites. For example, this approach has been used to monitor the prevalence of *T. b. brucei* and *T. b. rhodesiense* in domestic livestock following the Stamp Out Sleeping Sickness campaign in Uganda (see www.sleepingsickness.org).

Despite the reliability of these molecular techniques and their use as research tools, they are of no practical use as bed-side or pen-side diagnostic tools to direct treatment decisions. Nor are they useful for in-country monitoring. Several factors limit the usability of PCR in resource-poor field settings, including the need for a thermocycler and a reliable electricity source, as well as a gel tank and UV trans-illuminator for visualising the outcome of the reaction. Therefore there is a need to develop more practical methods for use in the field. Loop-mediated isothermal amplification (LAMP) is one such method that may prove practical for field-based molecular diagnostics.

1.3.5 Staging tools

If parasites are detected in the blood or lymph disease staging must be performed, which requires examination of the CSF. CSF is obtained by lumbar puncture. According to WHO recommendations [77] the patient is in stage 1 if the white cell count in the CSF is $\leq 5\ \mu l^{-1}$ and if trypanosomes cannot be observed. Stage 2 is characterised by the presence of trypanosomes and/or a white blood cell count of $\geq 20\ \mu l^{-1}$. There is an intermediate stage when trypanosomes are not observed and the cell count falls between these values. Patients in this category may or may not require stage 2 treatment.

Concentration techniques can be used to improve the sensitivity of trypanosome detection in the CSF. Centrifugation has been recognised to improve parasite detection since the early 1900s [110]. Double centrifugation (DC) was introduced in 1988 [111]. After the first centrifugation the sediment is taken up in micro-haematocrit tubes for a second centrifugation step. Although this increases the

sensitivity of detection it is not widely practised given the number of manipulations and the requirement for two centrifuges [112]. In 2000, the modified single centrifugation technique (MSC) was introduced [112]: 2 ml CSF is taken into a sealed Pasteur pipette (as prepared for mAECT) and centrifuged for 10 min at 600 g. The sediment is examined for trypanosomes by light microscopy using a mAECT viewing chamber. MSC is at least as sensitive as DC, and is easier to perform.

Staging is complicated. Most national programmes use different cell count thresholds, and may not consider an intermediate category. Thresholds are difficult to define and justify. Progress in defining new biological stage specific markers is being made [113, 114].

1.3.6 FIND and the search for new sleeping sickness diagnostics

FIND are working across all areas of sleeping sickness diagnosis including microscopy, sero-diagnosis, molecular diagnostics and disease staging tools.

1.3.6.1 Microscopy

Work done by FIND and their partners to improve mAECT has been previously mentioned. Also, in collaboration with Carl Zeiss (www.zeiss.com) FIND have also developed a simple LED based fluorescence microscope using acridine orange for parasite staining [115]. Finally, they have revived an old method using ammonium chloride for red blood cell lysis prior to centrifugation that may be useful for parasite concentration (www.finddiagnostics.org).

1.3.6.2 Serodiagnosis

FIND have recently signed an agreement with Standard Diagnostics Inc., Korea for the commercial development of rapid, point-of-care, dipstick style test for detection (but not discrimination) of *T. b. rhodesiense* and *T. b. gambiense*. This follows work done by FIND and partners to identify candidate antigens for detection of anti-trypanosome antibodies in patient sera. It is intended for screening in remote settings (http://www.finddiagnostics.org/media/press/100223.html; accessed 18th August 2010). In addition to FIND and their partners are also seeking novel antigen detection tests. Particularly, FIND is collaborating with the University of Brussels to look at nanobody technology and with the Seattle Biomedical Research Institute (SBRI) to apply single chain variable fragment (scFv) antibody engineering. See (www.finddiagnostics.org)

Nanobodies are small (15 kDa) antibody fragments derived from camelid conventional heavy chain antibodies. They bind antigen via one single chain variable domain, encoded by a 350 bp gene. They can be generated after immunisation of camelids with antigen, by purifying the camelid lymphocytes followed by RT-PCR, cloning in a phage display library and selection of the antigen specific antibodies. Once generated, they can be easily and economically produced and purified using bacterial, yeast or plant expression systems. They bind their target with high affinity and are small and stable with a long shelf life. It is relatively easy to produce larger bivalent or bispecific constructs, or nanobodies coupled to other protein carriers and they can be humanised by altering specific amino acid sequences to reduce the risk of inducing anti-nanobody antibodies [116, 117].

This technology has begun to be applied for the diagnosis of sleeping sickness. Stijlemans *et al.* [118] generated a nanobody which targets the conserved Asn-linked carbohydrate of trypanosome VSG and demonstrated its diagnostic potential for parasite detection with fluorescent microscopy. The entire surface of viable parasites

were stained, indicating that these small antibody fragments can penetrate the VSG coat to reach otherwise inaccessible constant epitopes.

More recently, Saerens *et al.* [119] generated several additional anti-VSG nanobodies using trypanosome infection of camelids (in place of antigen inoculation).

Two different antigen detection test formats are now under development using trypanosome specific nanobody technology. Firstly, a VSG specific dipstick format assay demonstrated an initial detection limit of 10 parasites ml^{-1} in a proof of principle study. Secondly, a novel diagnostic test using the PickPen (BioNobile) is also under development. This uses nanobody coated magnetic bead capture for antigen 'fishing' followed by detection using a second complementary nanobody [116].

Single-chain variable fragment (scFv) antibodies are one of the most popular recombinant antibody formats. Like nanobodies, they have many advantages in comparison to full length conventional monoclonal antibodies. In particular, their small size enables them to access targets which cannot be reaches by full size antibodies. They can also be produced economically, are open to genetic manipulation and have been engineered into larger, multivalent, bi-specific and conjugated forms [120]. FIND and SBRI are using yeast display systems to generate high affinity scFv for *T. brucei* proteins (http://www.finddiagnostics.org/export/sites/default/media/newsletters/old_issues/newsletter_jun_07.pdf; accessed 24th August 2010).

1.3.6.3 Molecular diagnostics

In October 2008 FIND signed a development agreement with Eiken Chemical Company, who originally developed LAMP (see below) to develop LAMP for sleeping sickness (http://www.finddiagnostics.org/media/press/081027.html; accessed 18th August 2010). To date FIND and their partners have been involved in the development of two LAMP assays for sleeping sickness, one targeting the *Trypanozoon* specific RIME element, and one targeting the *T. b. rhodesiense* specific *SRA* gene [121, 122]. Notably LAMP is the only isothermal DNA amplification technique that is being developed and evaluated by FIND for sleeping sickness. It is not the only isothermal DNA amplification technique that has been developed (see Section 1.5).

1.3.6.4 Disease staging tools

The methods for and difficulties associated with staging sleeping sickness were described in Section 1.3.5. At present staging requires investigation of the CSF, which is obtained by a painful and risky lumbar puncture procedure. To overcome this problem FIND and their partners have sought blood-based biomarkers for disease staging, but with little success. However, they have determined a panel of three brain damage marker proteins which can be used to discriminate stage 2, and could be of use in developing improved CSF staging tests [113]. FIND have also been involved in reformatting the latex/IgM card agglutination test into a more stable individual kit format for staging by CSF IgM quantification.

1.4 Loop-mediated isothermal amplification (LAMP): A novel diagnostic technology for use in developing countries?

Loop-mediated isothermal amplification (LAMP) is a DNA amplification technique developed in Japan by Eiken Chemical Company [123]. It is rapid, efficient and specific and has several advantages over traditional PCR. Most importantly, it does not require a high precision thermal cycler and gel-free amplicon detection systems can be used. Thus neither amplification nor detection requires equipment that would restrict its use to resource rich laboratory environments. As such it is being advocated as a suitable tool for low resource settings e.g. [124].

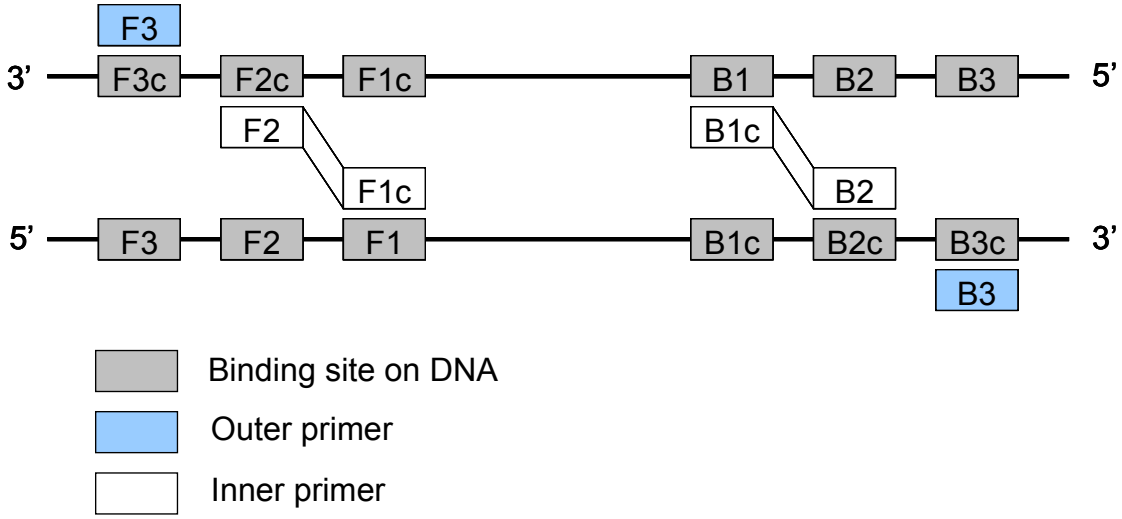
1.4.1 The LAMP reaction

1.4.1.1 Primers

LAMP requires a minimum of four primers, two inner primers and two outer primers, which recognise six distinct regions on the target sequence. The forward and backward outer primers, F3 and B3 respectively, are the same as regular PCR primers. F3 binds its complementary sequence (denoted F3c) on one strand of the DNA, while B3 binds its complementary sequence (denoted B3c) on the other strand. The inner primers each contain two segments and bind to both the sense and antisense strands of the target DNA. The forward inner primer (FIP) comprises two segments denoted F2 and F1c. The F2 segment binds to its complementary region (F2c) on the one strand, and the F1c segment binds to F1 region on the opposing strand. Likewise the backward inner primer (BIP) comprises two segments denoted B2 and B1c. This is depicted in Figure 1.1.

Figure 1.1. LAMP primers and their binding sites on the target DNA

Adapted from http://loopamp.eiken.co.jp/e/lamp/primer.html and 'A Guide to LAMP primer designing (PrimerExplorer V4)', Eiken Chemical Co. Ltd.



Once the inner and outer primers have been generated, two additional, optional 'loop primers' can be designed. These contain sequences complementary to the single stranded loop region in the starting material (see section 1.2.1.3. Mechanism). The forward loop primer (LF) binds between F1 and F2 and the backward loop primer (LB) binds between B1 and B2. This increases the number of starting points for DNA synthesis, and improves both the specificity and efficiency of the LAMP reaction [125].

1.4.1.2 Reaction conditions, reagents and templates

The reaction proceeds under isothermal conditions, typically between 60 and 65˚ C. It requires primers as described above, *Bst* DNA polymerase (which has strand displacement activity), betaine, buffer solution, template DNA and water to make up the reaction to a standard volume.

LAMP does not require purified DNA for efficient DNA amplification. In the literature LAMP reactions have been performed using extracted DNA and directly from heat treated blood samples, as well as CSF and native serum [126, 127]. This reduces the overall time and cost in comparison to systems in which DNA extraction is required. It has been suggested that *Bst* DNA polymerase is less susceptible than *Taq* to blood based PCR inhibitors such as heme [128]. LAMP may also be used with an RNA target by adding reverse transcriptase to the reaction mix. This is known as reverse transcription coupled LAMP (RT-LAMP) [129].

1.4.1.3 Mechanism

The mechanism of the LAMP reaction is summarised in Figure 1.2. It is also well described in an animation produced by Eiken Chemical Company (http://loopamp.eiken.co.jp/e/lamp/anim.html). It proceeds in two main stages: first the production of a stem loop structure and second the cycling amplification stage. It is dependent on the special design of the outer, inner primers and loop primers and upon the strand displacement activity of *Bst* DNA Polymerase.

Figure 1.2. The mechanism of the LAMP reaction

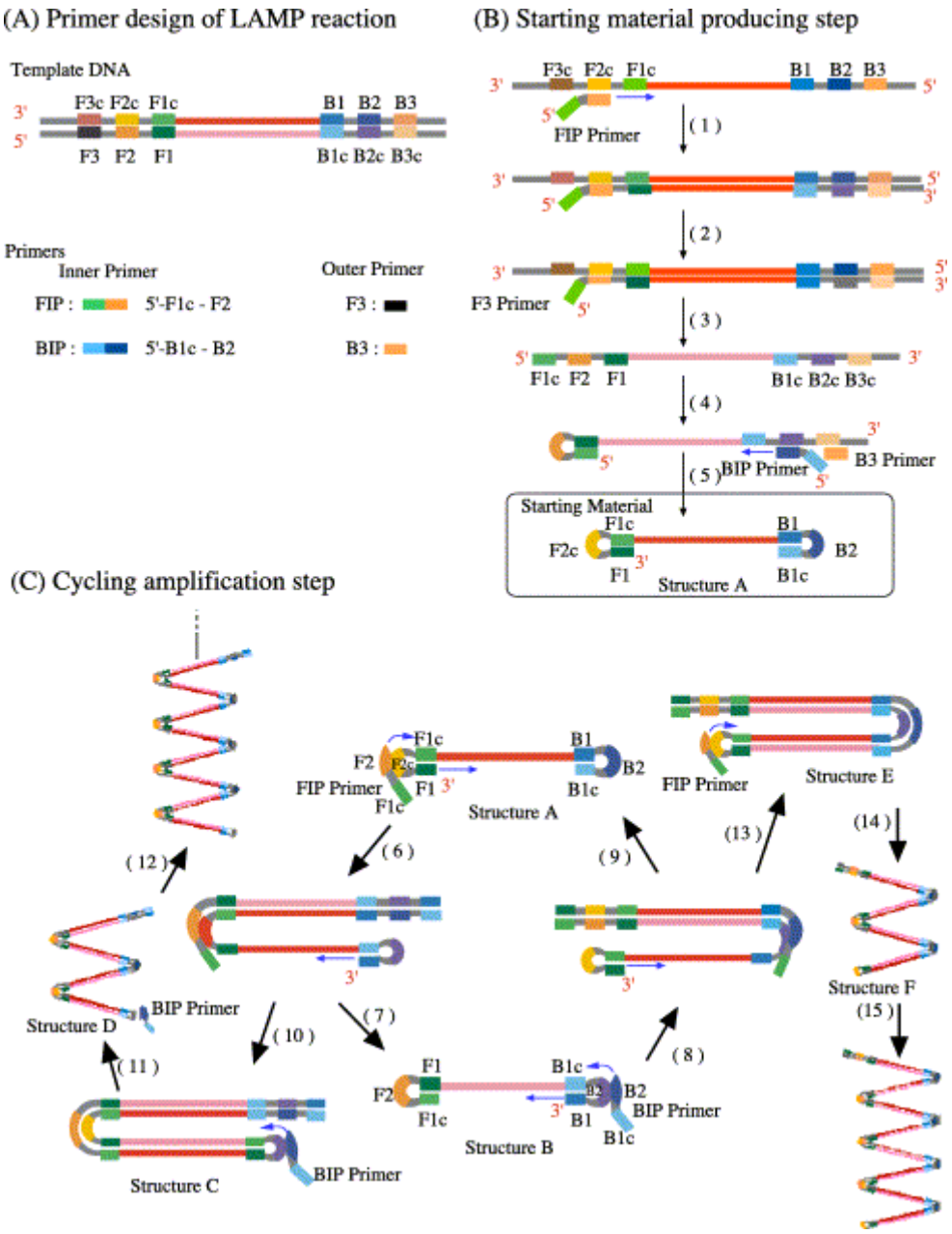


Figure taken from Mori *et al.* [130]

In the starting material producing step, the inner primers bind first to the target sequence. The outer primers are present at a much lower concentration in the reaction mix and so hybridise more slowly to the target sequence. Once an outer

primer is bound it initiates strand displacement DNA polymerisation generating a single stranded DNA, to which the other inner and outer primers bind (reactions 1-5 in Figure 1.2). This first stage generates a stem loop structure (Structure A) which acts as the starting material for the cycling amplification step. Once the starting material has been generated the outer primers play no further role in the reaction. During the cycling amplification step the inner primers bind the loop structure (reaction 6), generating a complementary stem loop (Structure B) and a new stem loop (reaction 10) whose stem is twice as long. A cyclic reaction between structures A and B is established (reactions 6-9). The elongated products (Structures C-F) are produced from the intermediate products formed during this cyclic reaction (reactions 10-12 and 13-15). The inner and loop primers bind the loop regions of the extended stem loops to generate these elongated products. Where loop primers are not included in the reaction strand displacement DNA synthesis is initiated from fewer loops at a time.

Since the target sequence is recognised by several independent sequences in all three stages of the reaction amplification is highly specific.

1.4.1.4 Detection

There are a variety of high and low technology methods in the literature for detecting positive LAMP amplification reactions. LAMP products may also be visualised directly by traditional gel electrophoresis and UV trans-illumination appearing as a ladder of bands [123]. Unlike PCR, for which the size of the amplicon can be used to confirm reaction specificity, the LAMP ladder indicates only that amplification has occurred, and provides no guarantee that the primers bound their intended target.

To confirm LAMP amplification of the correct target it is possible to sequence the LAMP product from a single band of the ladder excised after electrophoresis. Sequence specific visual detection of LAMP products is also possible using

fluorescently labelled DNA probes and low molecular weight polyethylenimene (PEI). A LAMP amplicon-PEI precipitate forms, incorporating the fluorescent labelled probes which can be visualised by UV light [131]. Finally endonuclease restriction and melting curve analysis e.g. [121] are sometimes used to confirm LAMP product specificity. Typically though, once a LAMP assay has been designed, optimized and validated positive amplification is assumed to be specific.

The simplest method of endpoint detection is optical turbidity assessment. Turbidity is derived from magnesium pyrophosphate formation as a by product of DNA polymerization, and is sufficient to be picked up by the naked eye for many LAMP reactions [124]. For a less subjective result this can be quantified by an endpoint turbidimeter, or monitored throughout by a real-time turbidimeter. Real time turbidimetry is particularly useful during assay design and optimization, enabling comparisons of the speed and efficiency of the reaction under different conditions.

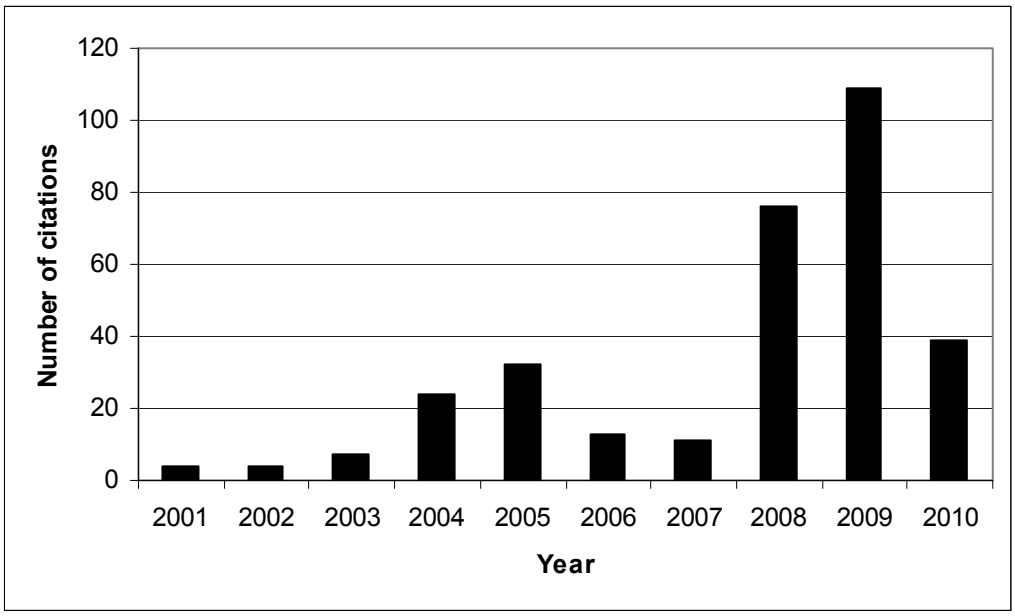
Several colour change methods for reading the result within the reaction tube have also been developed, including (i) DNA intercalating dyes: Quant-iT PicoGreen [132], SYBR green [133, 134] and propidium iodide [134] and (ii) metal ion indicator methods: calcein alone [135], calcein with $MnCl_2$ [136] and, most recently, hydroxynaphthol blue [137]. Metal ion indicators provide a simpler approach than the intercalating dyes; they are added alongside the other reagents, before incubation, so that amplification and detection are combined in a single processing step, within a closed tube system. The colour changes can be visualised by eye, without special lighting, and are inexpensive.

Methods which do not require the reaction tube to be opened after the amplication phase are favoured because they minimize the number of processing steps, and reduce the potential for laboratory contamination with LAMP products which would jeopardize the reliability of future tests.

1.4.2 Applications and uptake of LAMP technology

Since the first description of LAMP in 2000, there has been an explosion of LAMP publications describing its application as a molecular diagnostic for a wide range of pathogenic agents including *Mycobacterium tuberculosis*, *Plasmodium falciparum*, Influenza and measles viruses, *Salmonella* and *Eschericia coli* to name but a few [138]. The original publication describing the LAMP technique has been cited 319 times (on August 18th 2010), including 275 articles and 32 reviews.

Figure 1.3. Citations per year following the original publication of the LAMP method [123]



Analysis of these citations by subject area reveals LAMP has been applied for the detection of bacteria, viruses and parasites, as well as for other diagnostic purposes such as sexing. LAMP citations are found within the infectious disease, veterinary

sciences, plant sciences, tropical medicine, environmental health, food sciences, fisheries and marine biology literature.

In addition several kits have been developed by Eiken Chemical Company (see http://loopamp.eiken.co.jp/e/products/index.html). These kits are summarised in Table 1.4.

Table 1.4. Loopamp Detection Kits (developed and marketed by Eiken Chemical Company, Japan)

Field of use	**Specific application**
Environmental health	*Legionella* detection
Food safety	*Salmonella* detection
	Verotoxin-producing *Eschericia coli* detection
	Verotoxin typing
	Eschericia coli 0157 detection
	Listeria monocytogenes detection
	Campylobacter detection
Animal husbandry	Bovine embryo sexing
Research use only	Norovirus GI/GII detection

Despite this abundance of activity it remains to be seen whether this will translate into uptake and adoption in medical and veterinary public health and clinical practise. LAMP is being pursued as a potential clinical tool for tuberculosis and sleeping sickness diagnosis, especially by FIND (see section 1.3.6.3).

1.4.2.1 Applications of LAMP for identification of African trypanosomes

The LAMP method has already been applied to the detection of African trypanosomes. Successful reactions have been reported using purified DNA [139], treated blood, serum and cerebrospinal fluid (CSF) samples [127] and dried blood on filter papers [140]. Table 1.5 summarises the applications of LAMP technology to African trypanosomes.

Table 1.5. LAMP assays developed for the detection of African trypanosomes

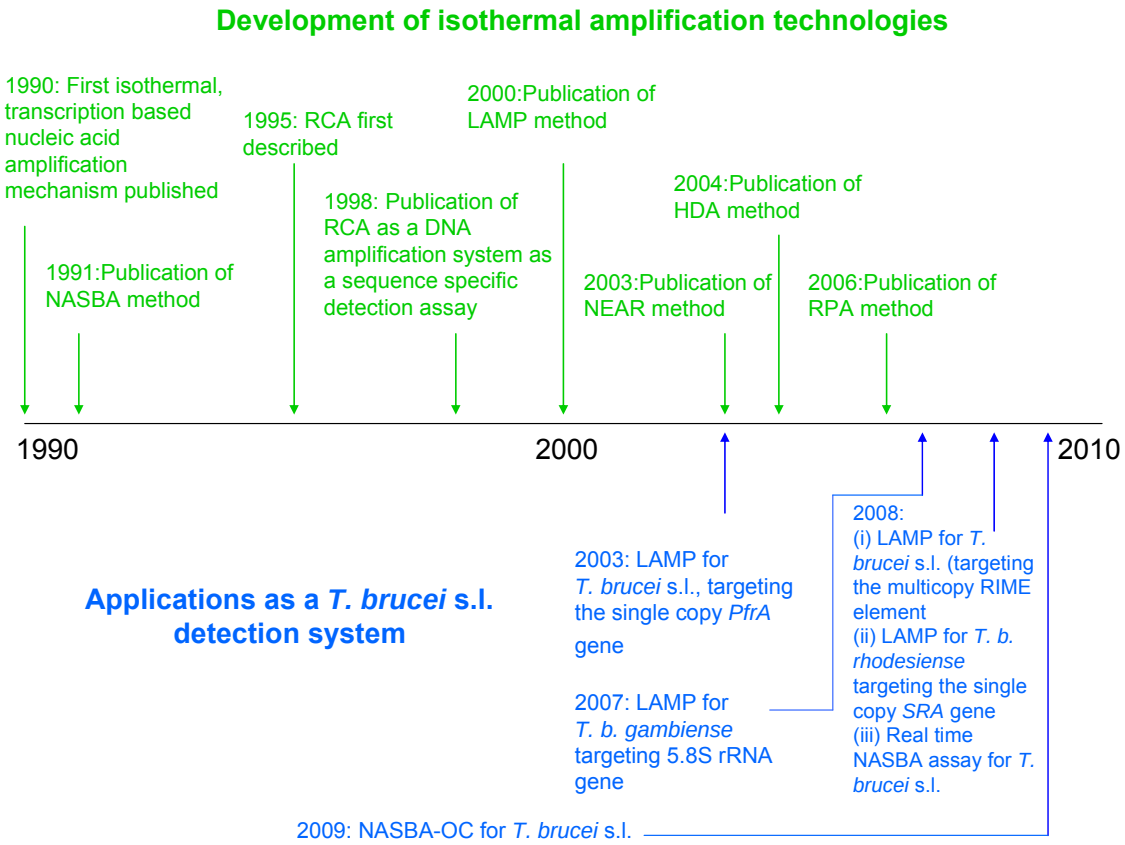
Parasite specificity	Target gene	Reference	Year
Trypanozoon	*PfrA* (paraflagellar rod protein A)	[140]	2003
Trypanozoon	RIME (repetitive insertion mobile element)	[127]	2008
T. b. gambiense	5.8S rRNA-ITS2	[141]	2007
T. evansi	RoTat1.2	[141]	2007
T. b. rhodesiense	*SRA* (serum resistance associated gene)	[122]	2008
T. congolense	*P0* (Ribosomal subunit)	[140]	2003
T. congolense	18S rRNA	[141]	2007

1.5 Other isothermal amplification techniques

Despite its prominence in the literature LAMP is not the only isothermal DNA amplification method to have been developed in recent years. Here the various methods are briefly described and compared, and some relevant applications are

highlighted. Many of these systems have been commercially developed into specific pathogen detection kits.

Figure 1.4. Timeline illustrating the development of isothermal amplification, and its application to *T. brucei* s.l.



1.5.1 Isothermal, transcription based amplification

In 1990 the first isothermal and one step transcription based nucleic acid amplification system was described [142]. The system generates antisense single stranded RNA from a single stranded RNA target via a cDNA intermediate using ribonuclease H, reverse transcriptase and DNA dependent RNA polymerase (enzymes normally used for retroviral replication). It was named 3SR.Ten million fold amplification occurs within 1-2 hours. The method has since been developed into the transcription mediated amplification (TMA) and the nucleic acid sequence based amplification (NASBA) approaches [143].

1.5.1.1 Transcription mediated amplification

The transcription mediated amplification (TMA) approach is a commercial development of the transcription-based system described above. Gen-Probe Inc. have developed several high through put TMA assays including the APTIMA COMBO 2 ® Assay for detection and differentiation of *Chlamydia trachomatis* and *Neisseria gonorrhoea* and the AMPLIFIED MTD ® Assay for *Mycobacterium tuberculosis*. Together with Novartis Vaccines and Diagnostics they have also developed TMA based assays for viral infections including the PROCLEIX ® HIV-1/HCV Assay, the PROCLEIX ® ULTRIO ® Assay for HIV-1, Hep C and Hep B and the PROCLEIX ® WNV Assay for West Nile Virus. Gen-Probe Inc have not made general purpose reagents commercially available, so at present the TMA approach is restricted to use of these kits by high throughput clinical and reference testing centres.

1.5.1.2 Nucleic acid sequence based amplification

The nucleic acid sequence based amplification (NASBA) method is also based on a transcription-mediated mechanism. It was first published in 1991 [144]. The three enzymes required (reverse transcriptase, RNAse H and T7 RNA polymerase) are available from bioMérieux, in their NucliSense basic kit. Since the start of this study NASBA has been applied (by others) to *T. brucei* s.l. [145, 146]. This assay is described as a potential competitor for LAMP based sleeping sickness diagnosis in Chapter 9.

1.5.2 Strand displacement amplification

The strand displacement amplification (SDA) system was first reported in 1992 [147, 148]. It has been developed into a high throughput DNA amplification assay for *Chlamydia trachomatis* and *Neisseria gonorrhoaea* by Becton Dickinson and Co. (BD ProbeTec™ ET System) for high volume molecular testing in large clinical and

reference laboratories (see http://www.bd.com/ds/productCenter/MD-ProbetecEt.asp)

Thus, SDA and TMA provide evidence that there is a market for, and acceptance of, high throughput isothermal amplification systems for molecular testing in large clinical and research laboratories, for some diseases. However, neither Becton Dickinson nor Gen-Probe Inc have opted to commercialise general purpose reagents, which limits the further development and application of these technologies for other infectious agents.

1.5.3 Rolling circle amplification

In 1995 small, single-stranded circular DNAs were first shown to behave as catalytic templates for DNA synthesis, in a process that became known as rolling circle amplification (RCA), which can occur under isothermal conditions [149, 150]. Subsequently this process was developed for sequence specific amplification detection [151, 152].

1.5.4 NEAR

NEAR is a patented technology belonging to Ionian Technologies, which was first described by the company's founder in 2003 [153] (see http://www.ionian-tech.com/index.html). It can be used to detect DNA or RNA targets, and the amplification products can be visualised using a variety of standard methods including real-time and endpoint fluorescence, agarose gel electrophoresis and mass spectroscopy. It proceeds at 60 ˚C and takes only 5 min. It has a single base resolution, with multiplex capabilities. Ionian Technologies are also developing a proprietary dipstick method for the detection of amplification products. According to their website, they are developing battery operated portable detectors for biothreat detection as well as rapid point of care diagnostics with lyophilised reagents stable at 37 ˚C and 42 ˚C, and requiring minimal sample preparation. In 2008, Ionian

Technologies were awarded a Bill & Melinda Gates Foundation grant to develop point-of-care diagnostic tests for the developing world with an initial focus on *Neisseria gonorrhoeae*, *Chlamydia* and *Mycobacterium tuberculosis*.

1.5.5 Helicase dependent amplification

Helicase dependent amplification (HDA) [154, 155] was developed by BioHelix Corporation (see www.biohelix.com). HDA proceeds by the same mechanism as PCR, except that a helicase enzyme is used instead of heat to melt the double stranded DNA. BioHelix Corp. have developed 2 product lines: (i) HDA-Inside which is a general purpose reagent and (ii) IsoAmp® On Demand Detection Kits for the detection of *Staphylococcus aureus* and *Staphylococcus aureus* mecA in research samples. Endpoint detection is via real-time flourescence detection. Therefore, although the amplification reaction itself is isothermal, isothermal incubation systems capable of real-time flourescence detection are not commercially available. In this format HDA does not constitute a low technology alternative to real-time, or traditional, PCR. However, real time detection is not necessary, and a light scanner could be used for endpoint detection. Work is also underway to develop a disposable lateral flow detection device into which a HDA reaction could be placed directly from a heating block. This would enable direct transfer of amplicon onto the strip in an enclosed setting, thus reducing the risk of laboratory contamination by the reaction products.

1.5.6 Recombinase polymerase amplification

The recombinase polymerase amplification (RPA) method was first published in 2006 [156]. It is a proprietary technology of TwistDx (see www.twistdx.co.uk). Unlike Gen-Probe Inc and Becton Dickinson and Co., TwistDx have commercialised general purpose reagents which can be applied by end users for specific assay development. These reagents are supplied in a dried stable format. Although TwistDx still recommend refrigeration for long-term storage, a strict cold chain is not required during transport, or in the short term. In addition TwistDx are also

developing pathogen specific detection kits for clinical diagnostics, biodefense and agriculture.

The reaction uses *Bacillus subtillis* Pol I (*Bsu*). DNA purification is not required, but amplification can be performed directly on crude samples, e.g. blood. RNA targets can also be amplified by addition of reverse transcriptase. The reaction proceeds at 37 ˚C and exponential amplification is achieved within 5-10 minutes. Several endpoint detection methods are possible, including standard gel electrophoresis, lateral flow strip detection and real-time and endpoint fluorescence using TwistDx fluorescent probe systems. This method has the capacity for multiplex detection.

1.6 Evaluating diagnostics

Before it can be usefully applied a diagnostic must be properly evaluated. However, concerns have been raised about the lack of rigorous and transparent evaluation for diagnostic tests, particularly those for use in developing countries. Many countries do not have regulatory standards for diagnostic tests, which means they can often be sold in the developing world without any formal evaluation of their performance and effectiveness [2, 3].

1.6.1 The evaluation process

Diagnostic evaluation is a several step process. Three distinct phases can be defined. In Phase 1 proof of principle studies are performed to provide evidence that the test detects the intended target. In Phase 2 the candidate test is evaluated in a case control study. These studies might make use of fresh patient, or archived samples. In Phase 3 the test is validated in the target population using a large-scale prospective study.

Several different characteristics need to be evaluated. First, test performance is critical. This is assessed by calculating the sensitivity and specificity, as well as the

positive and negative predictive values of a test. In addition to test performance the ease and conditions of use, the conditions of storage and the shelf life are key factors that must be evaluated. Finally, it is important to assess the reliability of any test.

1.6.1.1 Sensitivity and specificity

Sensitivity is the proportion of true positives that are detected. It represents the probability that the test will produce a true positive result when used on an infected population as compared to a reference or 'gold standard'.

Specificity is the proportion of true negatives that are detected by the test. It represents the probability that the test will produce a true negative result, compared to the 'gold standard', when used on a non-infected population.

Sensitivity and specificity are innate test characteristics that do not vary with prevalence. However, sensitivity and specificity estimates may vary among populations and subpopulations depending on the distribution of influential covariates. In practice sensitivity and specificity tend to be estimated as average values across non-homogenous populations. In reality, stratum-specific estimates might be more informative [5].

1.6.1.2 Predictive values

The positive predictive value (PPV) of a test is the probability that a person is infected when a positive test result is observed. The negative predictive value (NPV) is the probability that a person is not infected when a negative test result is observed. Unlike sensitivity and specificity, the predictive values of a test vary depending upon the prevalence of the condition being tested for in a specific population.

1.6.1.3 Kappa: a chance adjusted measure of agreement

Cohen's kappa statistic (κ) [157] can be used to quantify the level of agreement between the diagnostic and the gold standard. This gives a better indication of the concordance between the two techniques than percentage agreement, since it accounts for the agreement expected by chance.

1.6.1.4 Reliability

The reliability of a test can be defined as 'a measure of the extent to which replicate analyses using identical procedures agree with each other' [3]. Kappa, as described above, provides a useful measure for such agreement. Several different types of reproducibility might need to be evaluated. The intrinsic repeatability of the test itself is foremost. Once this has been established it is important to investigate reproducibility with different operators, at different test sites, with different kit lots and on different days. Ideally, reproducibility should be evaluated in a blinded study. The reliability of borderline diagnoses might be particularly important.

The calculation of sensitivity, specificity, positive and negative predictive value and Cohen's kappa statistic are described in the general methods (Chapter 2).

1.7 Identification and discrimination of the T. brucei s.l. sub-species: a historical overview

This thesis focuses on molecular detection of the human-infective African trypanosomes. It is therefore important to understand the background and discovery of key molecular markers in these organisms.

1.7.1 Discovery of the *T. brucei* s.l. subspecies

Trypanosoma brucei was discovered in 1895, among wild and domestic animals in Zululand, by Sir David Bruce [158, 159]. Trypanosome infection of man was later discovered in the blood of a steam boat captain on the River Gambia [160] and named *T. gambiense*. In 1903, trypanosomes were shown to be the causal agents of sleeping sickness with *Glossina palpalis* incriminated as the vector. Later, another human-infective trypanosome was identified from a sleeping sickness patient in Rhodesia [161], and named *T. rhodesiense*. These two were later reclassified as *T. b. gambiense* and *T. b. rhodesiense*. At first they were determined to be separate species based on morphological differences and transmission via different *Glossina* species [162]. However, they are now considered to be morphologically indistinguishable. At first *T. brucei* and *T. rhodesiense* were thought to be one species, however differences in the distribution of human and animal infections soon convinced many that these were different parasites [163]. Overtime several different experimental approaches have been used to determine the taxonomic relationship between these trypanosomes, and to find a consistent differentiation methodology.

1.7.2 The discovery and characterisation of the *SRA* gene for the identification of *T. b. rhodesiense*

From 1934 to 1955, during what was known as the Tinde experiment, *T. b. rhodesiense* was passaged through a series of experimental hosts over several years. The human infectivity of the passaged strains was periodically retested by inoculation into human 'volunteers' and proved to be an unstable, but persistent, characteristic of *T. b. rhodesiense* [164]. Following the Tinde experiment, in 1970, Rickman and Robson [165] developed the Blood Incubation Infectivity Test (BIIT) to assess the human infectivity of *T. brucei* s.l. strains. Using this test resistance to human serum was again shown to be an unstable characteristic which varies with strain passage [166]. In addition human serum resistance was shown to vary with the antigenic type of a given strain [167, 168].

It became possible to search for the difference between sensitive and resistant lines at a genetic level. By comparing the cDNA of human serum sensitive and serum resistant clones which expressed the same VSG a single, differentially expressed, gene was identified. This became known as the serum resistance associated, or *SRA* gene [24]. Later the same gene was independently isolated from a different *T. b. rhodesiense* strain [169].

SRA alone was shown to confer human serum resistance to *T. b. brucei* [170]. This together with an accidental infection of *T. b. brucei* transfected with the *SRA* gene [171] provided conclusive evidence for the role of *SRA* in human serum resistance in *T. brucei rhodesiense*. The mechanism by which the SRA protein confers this resistance was discovered later; in 2003 Vanhamme *et al.* demonstrated that SRA is a lysosomal protein whose amino terminal alpha helix interacts strongly with the carboxy terminal alpha domain of apolipoprotein L1 [28]. Since the *SRA* gene is found in a VSG expression site [170], it is only expressed when the resistance expression site (R-ES) is active. This explains the variability human serum resistance observed with antigenic variation.

The *SRA* gene appears to have arisen as a result of a 378 bp deletion within what was originally a VSG gene and the putative breakpoints for this deletion are conserved. [172, 173]. This has implications for primer design. Ideally primers should span the deletion site in order to be specific for *SRA* [108].

Since its discovery several PCR assays targeting *SRA* have been developed and have demonstrated its presence in all *T. b. rhodesiense* strains tested [33, 52, 54, 174-176]. At present there is no definitive marker for *T. b. brucei*, and therefore it is difficult to prove that *SRA* is absent in this species, however the *SRA* gene is conspicuously absent in strains known, or presumed, to be *T. b. brucei* [171]. Thus the *SRA* gene is

considered to be a ubiquitous and reliable diagnostic marker for *T. b. rhodesiense* as tested throughout its host and geographic ranges.

We now know that there are two main *SRA* variants; the 'northern' and 'southern' sequences [52, 54, 169, 172] which correlate with previously observed clinical differences [47, 54]. Despite slight variation between these sequences the *SRA* sequence is generally conserved across its geographic range, with less than 3 % sequence variation observed in isolates from disease foci spread across Ethiopia, Kenya, Tanzania, Uganda and Zambia [52]. The genomic position of the *SRA* gene is similarly conserved, downstream of *ESAG 5*, but again there is slight variation between the northern and southern *SRA* sequences.

The observed sequence and positional conservation contrasts with the extensive genetic variability seen with biochemical strain analysis. This has led to the suggestion that *SRA* arose once by a deletion event, and subsequently spread into different *T. b. brucei* genetic backgrounds by genetic exchange [171].

1.7.3 The discovery and characterisation of the *TgsGP* gene for the identification of *T. b. gambiense*

Following the discovery of the *SRA* gene it was proposed that another similar truncated VSG might similarly be responsible for human infectivity in *T. b. gambiense*. To test this hypothesis reverse transcriptase linked PCR (RT-PCR) was used to identify VSG like transcripts from *T. b. gambiense* LiTat 1.3 RNA. Two cDNAs carrying the 14 nucleotide VSG signature were obtained; regular VSG and a smaller transcript, subsequently named *TgsGP*. In the same publication the authors demonstrated that *TgsGP* encodes a 47kDa protein with a GPI-anchored VSG N-terminal domain, but without a typical VSG C terminal domain. They identified remarkable sequence similarity between amino acids 1-376 of *TgsGP* and the N-

terminal domain of *T. brucei* ILTat 1.23 VSG as well as between the next 27 amino acids of *TgsGP* and a C terminal section of *T. brucei* MITat 1.1 VSG [25].

However, *TgsGP* does not confer resistance to lysis by normal human serum. Although its exact function is unknown, several features suggest that it is a surface receptor which interacts with host components. It is surface exposed and concentrated at the flagellar pocket, has a similar structure to the trypanosome surface receptor, transferrin, and like other known components of the endocytotic machinery, is modified by the addition of pNAL [25].

In 2002 Radwanska *et al.* developed a PCR for the *TgsGP* gene [109]. Primers were designed avoiding the region which shares sequence similarity with the *T. b. brucei* IlTat 1.23 VSG gene and were carefully targeted to bind at the 3' end, where similarity to possible ancestral/ related genes is lost [109, 177]. Since the *TgsGP* sequence is well conserved in Group 1 *T. b. gambiense*, regardless of geographic origin, making it an ideal target for molecular detection of this parasite [177]. Using DNA extracted from trypanosome isolates the PCR was specific for conventional fully serum resistant *T. b. gambiense* (it successfully detected *TgsGP* in 13 Group 1 *T. b. gambiense* isolates, but not in two Group 2 *T. b. gambiense* or 58 other non *T. b. gambiense* isolates). The PCR was further validated using total DNA extracted from 92 sleeping sickness patients from Côte d'Ivoire. The results corresponded exactly to parasite detection by mAECT. A single round PCR could detect down to 1000 trypansomes ml^{-1} and this was improved to 10 trypanosomes ml^{-1} when a nested approached was used.

The assay was further developed and applied by Picozzi *et al.* [32], who developed a nested PCR protocol with additional outer primers, spanning the Radwanska target.

In humans *TgsGP* PCR has been used to investigate seropositive aparasitaemic patients. The *TgsGP* PCR was not consistent for these subjects and results did not agree with serological or parasitological detection. The utility of *TgsGP* PCR as a clinical diagnostic remains unclear. More fundamentally, the existence of a PCR positive, but otherwise indetectable reservoir of *T. b. gambiense* remains an unproven hypothesis, with significant potential epidemiological implications [45, 92].

TgsGP PCR has also been applied to animal populations, for example peridomestic livestock in Equatorial Guinea [178]. In this study, livestock were shown to harbour *T. b. gambiense* in some but not all disease foci. Again this has implications for our understanding of the epidemiology of Gambian sleeping sickness.

1.8 Prospects for point-of-care molecular diagnosis of sleeping sickness

Molecular diagnosis of Gambian and Rhodesian sleeping sickness is possible, by well established PCR assays, thanks to the discovery of the *SRA* and *TgsGP* genes. However, PCR is not suitable for point-of-care diagnosis. It is restricted to use in resource rich, often remote, research laboratories. LAMP is being promoted as a low technology DNA amplification technique suitable for the detection and identification of many and varied disease agents in low resource settings. LAMP assays for sleeping sickness have been published but have not been validated, either as epidemiological surveillance, or clinical diagnostic tools. There is a need to define the user requirements and performance standards for a high impact sleeping sickness diagnostic. If LAMP can fulfil these user requirements and performance standards it could be useful as a clinical diagnostic. LAMP may also be useful for remote epidemiological surveillance of sleeping sickness, as a cheaper, quicker, more sensitive alternative to PCR. The current trend for point-of-care diagnostics is towards a handheld or disposable device, with minimal to no additional

instrumentation requirements. In its current format LAMP technology does not meet these standards. However innovation is constant and many are striving to incorporate LAMP into a handheld device e.g. [179-181]. LAMP holds great potential as an improved molecular diagnostic for sleeping sickness.

With all of the above in mind the principle aims of this study are outlined below.

1.9 Thesis Aims

1. To determine whether LAMP assays for *Trypanozoon* detection might be a useful and simpler alternative to PCR as part of remote epidemiological surveillance studies of zoonotic parasites in the cattle reservoir.
2. To validate the published LAMP primers and assay for *T. b. rhodesiense* by comparison to the multiplex PCR for *SRA* as a reference test.
3. To design novel LAMP primers targeted to *SRA* accounting for its similarity to VSG genes.
4. To validate the published LAMP primers and assay for *T. b. gambiense* by comparison to the nested PCR for the *TgsGP* gene as a reference test.
5. To apply simple methods of LAMP endpoint detection to LAMP HAT assays.

2 Chapter 2: Materials and Methods

2.1 Samples

Three different types of DNA samples were used. These were: (i) commercially available human and cattle genomic DNA; (ii) DNA extracted from cryopreserved trypanosome isolates and (iii) DNA eluted from human and cattle blood stored on Whatman FTA cards.

2.1.1 Commercially available human and cattle genomic DNA

Genomic DNA from human placenta (D4642, Sigma, UK) and unsheared genomic DNA from calf thymus (D4764, Sigma, UK) were used as templates to check for cross reactions of trypanosome specific LAMP assays with host DNA. Dilution series in water were prepared from these DNA samples. For the human DNA 1 ml of water was added to 0.25 A260 units of DNA, to give a solution 0.0125 mg ml^{-1}. (One A260 unit is equivalent to 50 µg DNA). A tenfold dilution series was made and 1 µl of these dilutions was used to seed each LAMP reaction. By taking 1 µl of the 0.0125 mg ml^{-1} solution, 12.5 ng of DNA was added to the reaction mix.

For the cattle DNA 1 ml of water was added to 1 A260 unit of DNA. Given that there are approximately 20 A260 units per µg DNA, 1 unit is equivalent to 0.05 mg DNA. Therefore we have a 0.05 mg ml^{-1} solution. Taking 1 µl of this solution provides us with 50 ng DNA. A ten fold dilution series was made from this starting solution and 1 µl of these dilutions was used to seed each LAMP reaction.

2.1.2 Cryopreserved trypanosome samples

In total 86 samples were recovered from storage in liquid nitrogen. They were kept at -80 °C until processing (approximately 1 week). The sample set was chosen to include *Trypanosoma brucei brucei*, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. The isolates were either procyclic cultures frozen in Cunningham's media, with glycerol, or were bloodstream forms preserved in PSG Glycerol [182]. Although the majority of the isolates had been passaged through mice several times before their DNA was obtained, some were original isolates from the field. The samples were taken from cattle, humans, pigs and flies between 1968 and 1998. Individual sample details are given in Appendix 1. Twelve of these samples were taken from stocks that had been previously described as *T. b. gambiense* in the published literature (samples 5, 12, 17, 18, 19, 21, 22, 23, 58, 61, 62 and 75), of which nine belonged to Group 1 (samples 5, 12, 17, 18, 21, 22, 23, 58, and 62). A summary of the published isoenzyme analyses upon which the Group 1 and 2 identifications have been made can be found in Appendix 11.

In addition *T.congolense* ((Fly148) and *T. vivax* (TREU 1722) DNA that had been previously extracted from cryopreserved blood stream form trypanosomes using the Qiagen DNAeasy Blood and Tissue Kit (QIagen, UK), were used in Chapter 3 to assess the specificity of LAMP RIME and LAMP *PfrA*.

Frozen *T. b. brucei* (But135) and *T. b. rhodesiense* (DO) procyclic cultures were also used to obtain purified DNA for the initial screening of novel LAMP primers for *T. b. rhodesiense* (Chapter 4). But135 was isolated from a cow in Buteba village, Uganda in 1990 [183] while DO was isolated from a human patient in Southeast Uganda in 1990 [184].

2.1.2.1 DNA extraction from cryopreserved samples

DNA was extracted from the samples using a commercial kit (QIAamp DNA Blood Midi Kit, QIAGEN). First the samples were allowed to thaw at room temperature, before being transferred to screw-top tubes for washing and resuspension in phosphate buffered saline (PBS) as follows: centrifugation (3000 rpm, 5 minutes), removal of the supernatant and resuspension in 1 ml of PBS, followed by a second spin (3000 rpm, 5 minutes) and the final supernatant removal and pellet resuspension in 1 ml PBS. Pellets were not always visible, in which case only 80 % of the supernatant was removed and PBS was added to make the final volume of the sample back up to 1 ml.

The extraction protocol was performed according to the manufacturer's protocol. The cells within the prepared samples were lysed by adding the 1 ml samples to 100 µl of QIAGEN protease followed by 1.2 ml of Buffer AL. The mixture was shaken vigorously, incubated at 70 °C in a water bath for at least 10 minutes, 1 ml of ethanol was then added and the mixture shaken vigorously again. The sample DNA was then bound to the QIAamp Midi Column membrane by spinning the solution at 3000 rpm for 3 min, in a QIAamp Midi Column, placed in a 15 ml centrifuge tube. The filtrate was discarded and 2 ml of Buffer AW1 were added to the Midi column and the tubes were span at 5000 rpm for 1 min, before adding 2 ml Buffer AW2 and spinning again, at 5000 rpm for 15 min. Buffers AW1 and AW2 were both used to wash the DNA bound to the membrane and the filtrate from these two spin steps was discarded. The QIAamp Midi Columns were placed in fresh 15 ml collection tubes. Finally, to elute the DNA from the QIAamp midi column membrane, 200 µl of Buffer AE was pipetted onto the membrane and left to incubate at room temperature for 5 min. DNA was collected by spinning at 5000 rpm for 2 min. To maximize the DNA yield this elution process was then repeated, with fresh Buffer AE and a second spin step (5000 rpm, 2 min) before the DNA from the collection tube was transferred to a smaller, screw top tube, for transportation and storage. Samples were stored in the refrigerator. No special arrangements were made to keep the samples cool during

transport to Japan. During the flight they were transported in the hold and the journey was made during winter, so there was little opportunity for them to experience warm temperatures.

DO and But135 DNA, used in Chapter 4, were purified using the Qiagen DNeasy kit by following the manufacturer's protocol: 'Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol)' (Qiagen). First the stock solutions were allowed to thaw at room temperature and diluted by the addition of 1 ml of water. The sample was lysed by the addition of proteinase K, and the buffering conditions were optimised so that DNA bound onto the DNeasy mini spin column membrane during the subsequent centrifugation step. Two wash steps ensured the removal of contaminants from the membrane before the DNA was eluted.

2.1.2.2 Molecular characterisation of DNA extractions

The DNA concentration in each sample was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The 86 samples were then subject to the TCS PCR for the detection of *Trypanosoma congolense* savannah, TBR PCR for *Trypanozoon* detection, the nested *TgsGP* PCR for identification of *T. b. gambiense* and the multiplex *SRA* PCR for the identification of *T. b. rhodesiense* as described below.

In addition the ITS1 rDNA PCR [185] was performed to give a positive identification of the Fly 148 and TREU 1722 DNA samples used in Chapter 3 as *T. congolense* or *T. vivax*.

2.1.3 FTA card samples

Whatman FTA cards contain a chemically treated fibre matrix that lyses cells, inactivates proteins and immobilises DNA, making them suitable for long term

storage of blood samples. In this study both human and cattle blood samples that had been stored on FTA cards were used. Our standard laboratory protocol for preparation of these samples is described. It is based on the protocol published by Becker *et al* [186].

The first step in the preparation of these samples for PCR or LAMP was to take punches using either (i) a Harris Micro Punch (Whatman BioSciences Ltd) on a filter paper and cutting mat backing, or (ii) a Plier Craft Punch (Woodware).The number and diameter of the punches varied according to the particular experiment, as described in the methods sections of the subsequent chapters. Punches were placed in Eppendorf tubes. Five blank punches were made on filter paper between each sample and discarded, to avoid carry over of FTA card sample material between samples. Blank filter paper punches were also taken as negative controls for the FTA card preparation process.

Each set of punches was washed twice, with agitation, for 15 minutes each in 1 ml FTA purification reagent (Whatman) in order to remove blood intrinsic PCR inhibitors. This was followed by two further 15 min wash steps, with agitation, in 1 ml TE buffer (10 mM Tris-HCL pH 8.0; 1 mM EDTA pH 8.0) in order to remove the FTA purification reagent, which also inhibits the PCR reaction. The washed punches were transferred to open PCR tubes and allowed to dry for 30 min at 37 ˚C. DNA was eluted by adding 50 µl of 5 % (w/v) Chelex suspension to each sample, and heating at 90˚C for 30 minutes. Eluted DNA was stored at -20 ˚C. Five microliters of eluted DNA was used to seed a PCR or LAMP reaction.

2.1.3.1 FTA card samples used in Chapter 3

Two sets of cattle blood samples stored on Whatman FTA cards were used in Chapter 3. The first set were collected in 2008, as part of ongoing epidemiological studies on the prevalence of *T. brucei* s.l. in Uganda, during the Stamp Out Sleeping

Sickness campaign [187] and were used to compare LAMP *PfrA*, LAMP RIME and TBR PCR. These samples comprised 100 µl venous blood, collected from the ear of the cattle and stored on Whatman FTA cards. Blood was collected after cattle were cast to the ground by their owners. At the end of the day, or as soon as time permitted, the cards were stood out to dry before being packed into envelopes with silica gel desiccant for storage and transport.

The second set (105 sample**s**) were taken from our laboratory archives for checking the specificity of LAMP RIME and LAMP *PfrA*. They had been collected in Zambia in 1997 and 1998, and were stored on Whatman FTA cards. Previously species identification PCR assays had been performed for: (i) *T. congolense* savannah [188]; (ii) *T. congolense* forest [188]; (iii) *T. congolense* Kilifi [188]; (iv) *T. congolense* Tsavo [189]; (v) *T. vivax* (3 distinct PCR assays) [188, 190, 191]; (v) *Trypanozoon* (2 distinct PCR assays) [107, 192]. Each PCR assay had been seeded with a single 2 mm FTA card disc, which had been washed according to the usual protocol.

For both sets, five 3 mm punches were washed and dried and the DNA was eluted into 100 µl chelex (5 % w/v) suspension. DNA was prepared as described above. All PCR and LAMP reactions were seeded with 5 µl DNA eluted from the FTA card cattle blood samples.

2.1.3.2 FTA card samples used in Chapter 7

DNA was eluted from 52 human blood samples that were collected onto Whatman FTA cards between 2004 and 2006 from South East Uganda. Samples were collected from parasite positive patients (as determined by microscopy) as part of routine surveillance in conjunction with the Ugandan Ministry of Health. Three 2 mm punches were washed and dried according to usual protocol (Section 2.1.3) and the DNA was eluted into 100 µl chelex solution.

DNA was also eluted from 48 cattle blood samples collected onto Whatman FTA cards in central Uganda, October 2008 as part of ongoing monitoring of trypanosome prevalence in cattle for the SOS campaign (see www.stampoutsleepingsickness.com). Five 3 mm punches were taken, they were washed and dried according to usual protocol (Section 2.1.3) and the DNA was eluted into 100µl chelex solution.

2.2 PCR reactions

Four different standard PCR assays for the detection of *Trypanosoma congolense* savannah, *Trypanozoon*, *T. b. gambiense* and *T. b. rhodesiense* DNA were used throughout this work. All three are published methods and the primers are described in Table 2.1 below.

2.2.1 *Trypanosoma congolense* PCR reaction

PCR confirmation of *T. congolense* savannah DNA was made using the TCS PCR primers [188] which target a 369 bp satellite repeat sequence, first identified by restriction digest analysis and estimated to constitute approximately 5 % of the *T. congolense* nuclear DNA [107] . The reaction composition and cycling conditions were as follows: each 25 µl reaction contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 ^oC), 0.01% Tween-20, (collectively 10x NH_4 Reaction Buffer, Bioline), 1.5 mM $MgCl_2$ (Bioline), 1 U REDTaq (Bioline), 200 µM of each of the four dNTPs (Bioline), and 1 µM of each primer (TCS1 and TCS2). Thermal cycling was carried out as follows: 94 ^oC for 3 min followed by 30 cycles of 94 ^oC for 1 min, 55 ^oC for 2 min and 72 ^oC for 2 min, with a final extension step of 72 ^oC for 5 min. The expected band size was 316 bp.

2.2.2 *Trypanozoon* specific PCR reaction

Throughout this thesis PCR identification of *Trypanozoon* DNA was made using the TBR PCR primers [107] which target a 177 bp satellite repeat sequence [193, 194] of which there are 10000 copies per haploid genome. Twenty years after being first described in the literature this PCR assay remains the method of choice for the molecular detection of *Trypanozoon* parasites, having been successfully applied in several large-scale epidemiological studies [32, 195] using DNA from blood samples collected onto Whatman FTA cards. It is sensitive to as little as 0.01 pg of DNA.

The reaction composition and cycling conditions were as follows: each 25 µl reaction contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 ^oC), 0.01 % Tween-20, (collectively NH_4 Reaction Buffer, Bioline), 1.5 mM $MgCl_2$ (Bioline), 0.7 U REDTaq (Bioline), 200 µM of each of the four dNTPs (Bioline), and 0.8 µM of each primer (TBR1 and TBR2). Thermal cycling was carried out as follows: 94 ^oC for 3 min followed by 35 cycles of 94 ^oC for 1 min, 55 ^oC for 1 min and 72 ^oC for 30 s, with a final extension step of 72 ^oC for 5 min. The expected band size was 173 bp.

2.2.3 *T. b. gambiense* specific PCR reaction

PCR identification of *T. b. gambiense* was made using a nested PCR designed to target the *TgsGP* gene [32]. At the first round each 25 µl reaction contained 1.5 U HotStar Taq (Qiagen), 1 x NH_4 buffer (accompanying buffer for HotStar Taq (Qiagen)) containing 1.5 mM $MgCl_2$, 200 µM each of the four dNTPs (Bioline) and 1 µM each primer (*TgsGP*f1 and *TgsGP*r1). The reaction was initiated with a 15 min denaturation step at 95 ˚C, followed by 40 cycles of 94 ˚C for 40 seconds, 56 ˚C for 45 seconds and 72 ˚C for 45 seconds, and finally a 10 min extension step at 72 ˚C. The second round of PCR was always seeded with 1 µl of the first round product. Each 25 µl reaction contained 2.5 U HotStarTaq (Qiagen), 1 x NH_4 buffer with 1.5 mM $MgCl_2$, 5 µl of buffer Q (20 mM Tris-HCl pH 8.7, 100 mM KCl, 50 mM $(NH_4)_2SO_4$), 200 µM of each dNTP and 0.5 µM of each primer. This second round

reaction was initiated with a 15 min denaturation step at 95 ˚C, followed by 40 cycles of 94 ˚C for 1 min, 64 ˚C for 1 min and 72 ˚C for 1 min, and finally a 10 min extension step at 72 ˚C. These are in accordance with the published protocols, except that in the first round the extension step was left for 10 rather than 5 min and in the second round a 64 ˚C 1 min step was used instead of a 63 ˚C 1 min step. This was in line with the protocol used in our laboratory. The expected band size was 308 bp.

2.2.4 *T. b. rhodesiense* specific PCR reaction

The published multiplex PCR reaction for amplification of the GPI-*PLC* and *SRA* genes was used for the identification of *T. b. rhodesiense* [174]. A GPI-*PLC* band indicates the presence of sufficient genomic material for the amplification of a single copy gene and amplification of *SRA* is diagnostic of *T. b. rhodesiense*. The reaction was performed according to the published protocol. Each 25 µl reaction contained 1.5 U HotStar Taq, 1 x NH_4 buffer (accompanying buffer for HotStar Taq (Qiagen)) 1.25 µl Rediload dye (Invitrogen), 200 µM each dNTP, 3 mM $MgCl_2$ (Qiagen) and 0.2 µM each of four primers: SRA F, SRA R, PLC F and PLC R to amplify the *SRA* and *PLC* genes respectively. In some cases Rediload dye was not included, and the volume was made up to 25 µl using additional H_20. The reaction was initiated with a 15 min denaturation step at 95 ˚C, followed by 35 cycles of 94 ˚C for 30 s, 63 ˚C for 90 s and 72 ˚C for 70 s, and finally a 10 min extension step at 72 ˚C. The expected band sizes for this multiplex PCR are 324 bp for a PLC amplicon, > 1kb for a *VSG* amplicon and 669 bp for an *SRA* amplicon. For *T. b. rhodesiense* one would expect all three bands to be present. For other *T. brucei* s.l. the *VSG* and *PLC* bands alone are expected. The *PLC* band is an internal control to check that the quality and quantity of DNA in the sample is adequate for PCR amplification of a single copy gene.

2.2.5 ITS PCR

The ITS1 rDNA PCR [185] was performed to give a positive identification of the DNA samples as *T. congolense* or *T. vivax*. This single PCR can detect and identify all pathogenic trypanosomes. Each transcribed unit comprises 18S, 5.8S and 28S rRNA genes separated by two internal transcribed spacer regions [196-198]. The CF and BR primers bind to the 18S and 5.8S rDNA respectively for amplification the ITS1 region, which varies in size, enabling discrimination between trypanosome species [185]. Each 25 µl reaction contained 0.5 U BioTaq (Bioline, UK), 1 x NH_4 buffer (Bioline, UK) containing16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 ^oC), 0.01 % Tween-20, 2 mM MgCl2 (Bioline, UK), 0.4 µM CF and BR primers, 200 µM each dNTP and water. Thermal cycling was carried out as follows: 95 ^oC for 5 min followed by 35 cycles of 94 ^oC for 4 min, 58 ^oC for 30 s and 72 ^oC for 90 s, with a final extension step of 72 ^oC for 2 min. A 700 bp band was expected for *T. congolense* savannah or forest, 250 bp for *T. vivax* and 480 bp for any member of the sub-genus *Trypanozoon*.

2.2.6 Identifying PCR positive reactions

Reaction products were held at 4 ˚C pending analysis. The reactions products were analysed by gel electrophoresis and UV illumination to detect the presence of an appropriately sized band corresponding to the size of the amplified fragment of DNA. Gel electrophoresis was performed at 100 V in a 1% TBE-agarose gel containing either ethidium bromide or GelRed (Biotium). The use of GelRed replaced ethidium bromide in our laboratory during the course of this work because it is a safer and more sensitive alternative that can be used with the same imaging system. For those reactions in which Rediload dye was not included in the reaction mix Gel Loading Buffer (Sigma) was mixed with the reaction products prior to loading into the gel.

Table 2.1. PCR primer sequences used for identification of *Trypanosoma congolense* savannah, *Trypanozoon* DNA and species specific identification of *T. b. gambiense* and *T. b. rhodesiense*

PCR assay specificity	Target gene	Primer	Sequence
All pathogenic trypanosomes	ITS	CF	CCGGAAGTTCACCGATATTG
		BR	TTGCTGCGTTCTTCAACGAA
T. congolense savannah	316 bp satellite	TCS1	CGAGAACGGGCACTTTGCGA
		TCS2	GGACAAACAAATCCCGCACA
Trypanozoon	177 bp satellite	TBR1	CGAATGAATATTAAACAATGCGCAGT
		TBR2	AGAACCATTTATTAGCTTTGTTGC
T. b. gambiense	*TgsGP*	*TgsGP*f1	GATGTGCTGCTTTGCGTATG
		*TgsGP*r1	GGGCCTTCACATAAGAATT
		*TgsGP*f2	GCTGCTGTGTTCGGAGAGC
		*TgsGP*r2	GCCATCGTGCTTGCCGCTC
T. b. rhodesiense	*SRA*	SRA F	GAAGAGCCCGTCAAGAAGGTTTG
		SRA R	TTTTGAGCCTTCCACAAGCTTGGG
	PLC	PLC F	CGCTTTGTTGAGGAGCTGCAAGCA
		PLC R	TGCCACCGCAAAGTCGTTATTTCG

2.3 LAMP reactions

Four different published LAMP assays for the detection of *Trypanozoon*, *T. b. gambiense* and *T. b. rhodesiense* DNA were used throughout this work and are described in more detail below. The primer sequences are given in Tables 2.2, 2.3 and 2.4 below.

2.3.1 *Trypanozoon* specific LAMP

Two *Trypanozoon* specific LAMP reactions have been used in this study, one targeting the single copy paraflagellar rod protein A (*PfrA*) gene (henceforth referred to as LAMP *PfrA*) [140] and a second targeting the multicopy RIME element (henceforth referred to as LAMP RIME) [121].

For LAMP *PfrA* each 25 µl reaction contained 8 U *Bst* DNA polymerase (New England Biolabs), 2.5 µl Thermopol reaction buffer I with additional $MgSO_4$ (New England Biolabs) to give 20 mM Tris-Cl, 10 mM KCl, 10 mM $(NH_4)SO_4$, 0.1 % Triton X-100 and 8 mM $MgSO_4$, 0.8 M betaine (Sigma Aldrich), 1.4 mM of each of the four dNTPs (Bioline), 1.6 µM of both FIP and BIP and 0.2 µM of both F3 and B3, the LAMP primers. When performed in a thermal cycler, or simple heating block, the reaction mix was incubated at 65 °C for 1 hour followed by termination at 80 °C for 2 min. Reaction products were then held at 4 °C until analysis. When the reaction was performed in the Loopamp Real Time Turbidimeter (Eiken Chemical Co. Ltd.) the reaction was simply held at 65 °C for 90 min. Note that the detergent (Triton X-100) in the reaction buffer is different to the detergent in the reaction buffer used by the original authors (Tween 20).

For LAMP RIME each 25 µl reaction contained 8 U *Bst* DNA polymerase (New England Biolabs), 2.5 µl Thermopol reaction buffer I (New England Biolabs) to give 20 mM Tris-Cl, 10 mM KCl, 10 mM $(NH_4)SO_4$, 0.1 % Triton X-100 and 2 mM $MgSO_4$, 0.8 M betaine (Sigma Aldrich), 200 µM of each of the four dNTPs (Bioline), 2 µM of both FIP and BIP, 0.2 µM of both F3 and B3 and 0.8 µM of both LF and LB. When performed in a thermal cycler, or simple heating block, the reaction mix was incubated at 62 °C for 1 h followed by termination at 80 °C for 4 min. This follows the published protocol exactly. Reaction products were then held at 4 °C until analysis. When the reaction was performed in the Loopamp Real Time Turbidimeter (Eiken Chemical Co. Ltd.) the reaction was simply held at 62 °C for 90 min.

2.3.2 *T. b. rhodesiense* specific LAMP

When this work started in 2008 and at the time of writing a single published LAMP reaction designed to identify *T. b. rhodesiense* could be found in the literature [122]. This assay was performed in the current work according to this published protocol. Each 25 µl reaction contained 8 U *Bst* DNA polymerase (New England Biolabs), 2.5 µl Thermopol reaction buffer I (New England Biolabs) to give 20 mM Tris-Cl, 10 mM KCl, 10 mM $(NH_4)SO_4$, 0.1 % Triton X-100 and 2 mM $MgSO_4$, 0.8 M betaine (Sigma Aldrich), 200 µM of each of the four dNTPs, 2 µM of both FIP and BIP, 0.2 µM of both F3 and B3 and 0.8 µM of both LF and LB. When performed in a thermal cycler, or simple heating block, the reaction mix was incubated at 62 °C for 1 h followed by termination at 80 °C for 4 min. Reaction products were then held at 4 °C until analysis.

2.3.3 *T. b. gambiense* specific LAMP

Similarly, one published LAMP assay designed to identify *T. b. gambiense* could be found in the literature [141]. In the current work this assay was performed according to the published protocol, except for the use of Triton X-100 containing buffer in place of Tween-20 containing buffer. All other buffer constituents were identical. Each 25 µl reaction contained 8 U *Bst* DNA polymerase (New England Biolabs), 2.5 µl Thermopol reaction buffer I with additional $MgSO_4$ (New England Biolabs) to give 20 mM Tris-Cl, 10 mM KCl, 10 mM $(NH_4)SO_4$, 0.1 % Triton X-100 and 8 mM $MgSO_4$, 0.8 M betaine (Sigma Aldrich), 1.4 mM of each of the four dNTPs, 1.6 µM of both FIP and BIP and 0.2 µM of both F3 and B3. When performed in a thermal cycler, or simple heating block, the reaction mix was incubated at 63 °C for 1 h followed by termination at 80 °C for 2 min. Reaction products were then held at 4 °C until analysis. When the reaction was performed in the Loopamp LA-200 real time turbidimeter (Eiken Chemical Co. Ltd.) the reaction was simply held at 63 °C for 90 min.

Table 2.2. LAMP primer sequences used for identification of *Trypanozoon* DNA

Target gene	Primer	Sequence
PfrA	FIP	TCAGAAGCGTCGAGCTGGGATTTTATCGACAATGCCATCGCC
	BIP	CGCAAGTTCCTGTGGCTGCATTTTTTCCCAAGAAGAGCCGTCT
	F3	TCACAACAAGACTCGCACG
	B3	GGGCTTTGATCTGCTCCTC
RIME	FIP	GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA
	BIP	AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC
	LF	CTGTCCGGTGATGTGGAAC
	LB	CGTGCCTTCGTGAGAGTTTC
	F3	GCCTCCCACCCTGGACTC
	B3	AGACCGATAGCATCTCAG

Table 2.3. LAMP primer sequences used for identification of *T. b. rhodesiense*

Target gene	Primer	Sequence
SRA	FIP	GGACTGCGTTGAGTACGCATCCGC AAGCACAGACCACAGC
	BIP	CGCTCTTACAAGTCTTGCGCCCTTCTGAGATGTGCCCACTG
	LF	GCGGAAGCAAGAATGACC
	LB	TCTTACCTTGTGACGCCTG
	F3	CGCGGCATAAAGCGCTGAG
	B3	GCAGCGACCAACGGAGCC

Table 2.4. LAMP primer sequences used for identification of *T. b. gambiense*

Target gene	Primer	Sequence
5.8S rRNA-ITS 2	FIP	GCGTTGAACAACACAAAATAGGTGATGCCACATTTC TCAGTGT
	BIP	CCACCTCTTCTCCTCGTGTGGAAGAAAGAGATGAAA GATATCGTA
	F3	AAGCTCTCTCGAGCCATC
	B3	TGACATACACAATATGTGCGA

2.3.4 Detecting LAMP products

DNA amplification was assessed by electrophoresis in a 1% TBE - agarose gel containing either ethidium bromide or GelRed. The use of GelRed replaced ethidium bromide in our laboratory during the course of this work because it is a safer and more sensitive alternative that can be used with the same imaging system. Gel

Loading Buffer (Sigma) was mixed with the reaction products prior to loading into the gel. Reaction products were seen as a ladder of multiple bands under UV illumination, indicating the production of stem-loop DNAs containing varying numbers of inverted repeats of the target sequence. This usually had a smeared appearance.

Other LAMP product detection methods included

(i) the turbidity of the product in the reaction tube observed by eye

(ii) real time turbidimetry using the time to reach a threshold turbidity measurement of 0.1 [130]. This is a standard value chosen to define a positive reaction in a real time turbidimeter e.g. [133].

(iii) colour change with Quant-iT PicoGreen. 2 µl or 5 µl is added post-incubation. Colour is then observed by eye under normal light, and fluorescence is observed under UV light

(iv) colour change with hydroxynaphthol blue. Before incubation 120 µM hydroxynaphthol blue was included in each 25µl reaction mix. Upon termination colour was assessed by eye, under normal light.

(v) colour change with calcein and $MnCl_2$. 25 µM calcein and 0.5 mM $MnCl_2$ were included in each 25 µl reaction mix before incubation. Upon termination the colour was assessed by eye, fluorescence under UV observed

(vi) 1 µl Loopamp Flourescence Detection Reagent (Eiken, Japan) added to the reaction mix prior to incubation. An orange to green colour change was assessed by eye, and the fluorescence under UV was observed.

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2.4 Novel LAMP primer design

LAMP primers are designed against a target sequence. LAMP requires a minimum of four primers, two inner primers and two outer primers, recognising six distinct regions on the target sequence. The forward and backward outer primers, F3 and B3 respectively, are the same as regular PCR primers. The inner primers each contain two segments and bind to both the sense and antisense strands of the target DNA. The forward inner primer (FIP) comprises two segments denoted F2 and F1c. Likewise the backward inner primer (BIP) comprises two segments denoted B2 and B1c. This is shown in Figure 2.1.

Once the inner and outer primers have been generated, two additional, optional 'loop primers' can be designed. These contain sequences complementary to the single stranded loop region in the starting material. The forward loop primer (LF) binds between F1 and F2 and the backward loop primer (LB) binds between B1 and B2. Loop primers are not required for LAMP amplification, but they can improve both the specificity and efficiency of the reaction [125] by increasing the number of starting points for DNA synthesis.

After the choice of an appropriate target sequence there are four key factors in the design of successful LAMP primers: the melting temperature (T_m), stability at the primer ends, GC content and secondary structure. The distance between primers is also important. The importance of these factors, and their incorporation into the design process is discussed in 'A Guide to LAMP primer designing (PrimerExplorer V4)' produced by Eiken Chemical Co. Ltd and available through their website. LAMP primers can be designed using the PrimerExplorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html) which automatically accounts for each factor. This requires a minimum length target sequence of 200 bp.

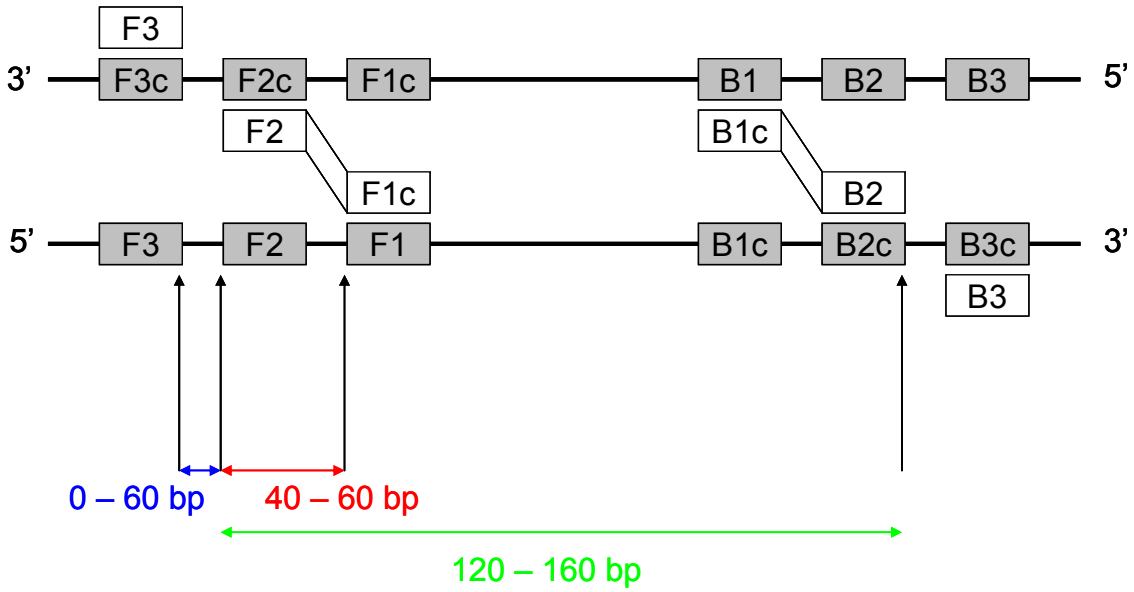
Using the PrimerExplorer V4 software the T_m is designed to be in the range 64-66 ˚C for F1c and B1c, 59-61 ˚C for F2, B2, F3 and B3 and 64-66 ˚C for the loop primers. The software uses the nearest-neighbour method to calculate T_m, for standard experimental conditions of 0.1 μM oligos, 50 mM Na^{+} ions, 4 mM Mg^{2+} ions. However these conditions are often changed during LAMP primer assay optimization.

The stability of the primer ends is important because they provide the starting point for DNA synthesis. Hence, the primers are designed to so that the 3' ends of F2, B2, F3, B3, LF and LB and the 5' ends of F1c and B1c have a free energy change (ΔG) -4 kcal mol^{-1} or less. The more negative the ΔG, the more often the primer anneals to the template.

The GC content of the primers is designed to be in the range 40-65 %. Primer design also aims to prevent the formation of secondary structures and primer dimers. The distance between the 5' ends of F2 and B2 should be in the range 120-160 bp, the distance between the 5' ends of F2 and F1 (or B2 and B1) should be in the range 40-60 bp and the distance between the 3' end of the outer primer and the 5' end of the inner primer should be between 0-60 bp.

Most simply the Primer Explorer V4 software designs primers across the whole target region. However, the user can specify the binding site of any given primer. In addition mutations on the target sequence can be highlighted in order that specific, or common, primers can be designed.

Figure 2.1. LAMP primers and their binding sites on the target DNA



Adapted from http://loopamp.eiken.co.jp/e/lamp/primer.html and 'A Guide to LAMP primer designing (PrimerExplorer V4)', Eiken Chemical Co. Ltd

2.4.1 LAMP primer design for novel *T. b. rhodesiense* specific assays

Two methods were used to design the LAMP primer sets. Firstly the location of F1, F2, F3, B1, B2 or B3 was fixed to bind from position 571-590. For each, the automatic judgment parameter set and the default design options were selected. First the basic design option was used. When this did not generate any primer sets the AT rich parameter setting was selected within the detailed settings. Since this generated numerous primer sets the easy sorting rule was applied to select the best of the candidate sets. Primer information files were used to design loop primers. Where several loop primer sets were generated the first in the list was selected for testing (see summary Tables 2.5 and 2.6).

Table2.5. Design process for *SRA* specific LAMP primers

ID	Primer region fixed at position 571-590	Number of primer sets generated with:		
		(i) Basic design option	(ii) Detailed settings; AT rich parameter; no sorting rule	(iii) Detailed settings; AT rich parameter; easy sorting rule
SRALAMP_a	B3	0	1000	2
SRALAMP_b	B1	1	Not done	Not done
SRALAMP_c	F3	0	549	2
SRALAMP_d	F2	0	234	1
SRALAMP_e	B2	0	108	1
SRALAMP_f	F1	0	398	1

Table 2.6 Loop primer design for *SRA* specific LAMP primers

ID	Number of loop primers generated		
	LF	LB	Sets
SRALAMP_a-[6]	17	63	222
SRALAMP_a-[1]	7	63	60
SRALAMP_b	0	0	0
SRALAMP_c-[6]	1	64	0
SRALAMP_c-[1]	30	12	312
SRALAMP_d	0	15	0
SRALAMP_e	12	27	27
SRALAMP_f	42	12	41

Secondly primer design software was used to design specific primers to recognise a mutation at position 581. Again automatic judgment setting was selected. The mutation was set at position 581. Using basic design settings, no primer sets were generated but when the AT rich parameter was chosen 18 primer sets were generated. The recognition site was located at the 5' end of the F1c segment for six sets, and at the 3' end of the F2 segment for 12 sets.

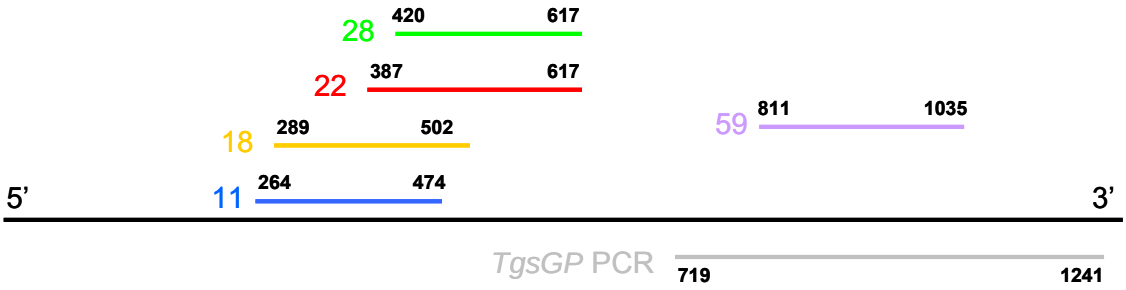
The primer sets were compared (Appendix 6) and five were selected for testing (SRALAMP7.1, SRALAMP7.2, SRALAMP7.3, SRALAMP7.10 and SRALAMP7.13) - where primer sequences were identical or very similar they were not ordered in duplicate. Primers sets were constructed using some common primers and differ slightly from the primer sets generated (Appendix 6, Table 3). No loop primers sets could be designed for any of these primer sets.

The sequences and binding sites of all primers in all novel primer sets designed, in relation to the position of the *SRA* specific internal deletion, are shown in Appendix 7.

2.4.2 LAMP primer design for novel *T. b. gambiense* specific assays

Primers were designed using the *TgsGP* sequence with accession number AJ277951. Five sets of LAMP primers were generated using the automatic judgement default setting of the primer design software. No modifications were made to any of the parameter settings. Five sets of LAMP primers were generated (see Appendix 14). Primers sets 22, 11, 18 and 28 bind towards the front end of the *TgsGP* sequence whereas primer set 59 binds in the same region of the gene as the nested *TgsGP* PCR primers (shown in Appendix 15 and summarised in Figure 2.2 below).

Figure 2.2. Relative target regions of the *TgsGP* PCR [75] and LAMP primers within *TgsGP* gene AJ277951



Primer set 59 was selected for further investigation as it was considered less likely to give rise to non-specific amplification with other *T. brucei* VSG genes (henceforth referred to as LAMP *TgsGP*). The sequences and other key characteristics of the *TgsGP* LAMP primers are summarised in Table 2.7. The binding sites of all LAMP *TgsGP* primers relative to *TgsGP* PCR primers are shown in more detail in Appendix 15.

Table 2.7. Characteristics of the LAMP *TgsGP* primers

Primer	5' to 3' position	Length	Tm	5' ΔG	3' ΔG	GC rate	Sequence
F3	811-829	19	59.44	-5.26	-4.42	0.58	GAAGCAGTGGGACCTTAGC
B3	1081-1035	18	60.89	-4.18	-7.36	0.56	TTAACGCAGACACCGCCT
FIP		40					GATCCCGCCTGCTGTGGTTTT-CGATGTCGCTGCTGTGTTC
BIP		40					TCCAAATTGACGGGGACAACGG-GCCGTTCCTGTGCAGTTG
F2	838-856	19	60.79	-5.23	-4.67	0.58	CGATGTCGCTGCTGTGTTC
F1c	896-916	21	65.33	-5.20	-4.27	0.57	GATCCCGCCTGCTGTGGTTTT
B2	997-1014	18	60.74	-6.68	-4.66	0.61	GCCGTTCCTGTGCAGTTG
B1c	948-969	22	65.10	-4.53	-5.94	0.55	TCCAAATTGACGGGGACAACGG

2.5 Development of novel LAMP assays

2.5.1 Initial trial of primers for *T. b. rhodesiense*

Nine primer sets were selected for the initial trial (Table 2.8). All novel primers designed for the *SRA* gene were initially tested under the following reaction conditions: 62 ˚C for 1 h followed by a 4 min termination step at 80 ˚C and held at 4 °C using a thermocycler until analysis. A reaction volume of 25 µl was used with 1 µl template DNA and containing 8 U *Bst* DNA polymerase (New England Biolabs), 2.5 µl Thermopol reaction buffer I (New England Biolabs) to give 20 mM Tris-Cl, 10 mM KCl, 10 mM $(NH_4)SO_4$ and 2 mM $MgSO_4$, 5 µl Q buffer (Qiagen), 200 µM dNTPs, 2 µM of both FIP and BIP, 0.2 µM of both F3 and B3 and 0.8 µM of both LF and LB. Additional $MgSO_4$ was used to adjust the final concentration to test these primer sets over a range of $MgSO_4$ concentrations (Table 2.8). Where loop primers

were not included water was added to make up the reaction volume, all other conditions were kept the same.

Initial screening of the novel primers was carried out using purified DNA extracted from frozen stocks of parasites But135 (*T. b. brucei*) and DO (*T. b. rhodesiense*).

Table 2.8. Summary of primer sets trialled for *SRA* specific LAMP

Primers	Region containing deletion site	Loop primers?	[$MgSO_4$]
SRALAMP_a	B3	Yes	2 mM
SRALAMP_b	B1	No	2, 4, 6 and 8 mM
SRALAMP_e	B2	Yes	2, 4, 6 and 8 mM
SRALAMP_f	F1	Yes	2, 4, 6 and 8 mM
SRALAMP7.1	F1c	No	2, 3, 3.5, 4, 4.5 and 5 mM
SRALAMP7.2	F2	No	2, 4, 6 and 8 mM
SRALAMP7.3	F2	No	2, 4, 6 and 8 mM
SRALAMP7.10	F1c	No	2, 4, 6 and 8 mM
SRALAMP7.13	F1c	No	2, 4, 6 and 8 mM

2.5.2 Adaptation of the novel assay for *T. b. rhodesiense* for use with the real time turbidimeter

Three modifications were made to the LAMP reaction mix for use with the real time turbidimeter. LAMP buffer was altered to contain Tween-20 in place of Triton X-100, the concentration of $MgSO_4$ was increased from 2 mM to 8 mM and the concentration of dNTPs were simultaneously increased from 0.2 mM to 1.4 mM. These changes were tested using four *SRA* PCR positive samples (32, 36, 64, 65 and

68) and two *SRA* negative samples (24 and 28) and the changes were tested for LAMP reactions run using both heating block followed by gel electrophoresis, and using a real time turbidimeter.

2.5.3 Application of the novel assay for *T. b. rhodesiense* to the complete sample set using a heating block

The SRALAMP_a assay was also applied to the 86 samples using a thermocycler to incubate the reaction at 62 ˚C for 60 min, followed by a 4 min 80 ˚C termination step. The reactions were then held at 4 ˚C until they were assessed. Assessment was made by optical turbidity assessment, and by gel electrophoresis with subsequent UV illumination for all samples. This full sample set screen was performed three times. For these reactions the reaction mix was the same as with the real time turbidimeter screen described above, except that the reaction buffer contained 0.1 % Triton X-100 in place of 0.1 % Tween-20. The reactions were seeded with 1 µl of the DNA sample.

2.5.4 Application of the novel assay for *T. b. rhodesiense* to the complete sample set using the real time turbidimeter

The SRALAMP_a assay was applied to all 86 samples using a real time turbidimeter. The reaction mix contained 8 U *Bst* DNA polymerase, 1 X LAMP buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween-20), plus additional $MgSO_4$ to give a final concentration of 8 mM $MgSO_4$ 0.8 M betaine, 1.4 mM dNTPs, 2 µM FIP and BIP, 0.8 µM LF and LB, 0.2 µM F3 and B3 and H_20 to make the reaction volume up to 25 µl. The temperature was set at 62 ˚C for 90 min, and the time to a threshold turbidity of 0.1 was noted. The reactions were seeded with 1 µl of the DNA sample.

2.5.5 Optimisation of reaction conditions including loop primer concentration and temperature

To see whether the time to reach the turbidity threshold could be reduced, and to make the reaction as sensitive as possible, loop primer concentration and incubation temperature were tried over a range of reasonable values.

Since loop primers can improve LAMP reaction efficiency, the SRALAMP_a assay was trialled without the loop primers, with the loop primers 0.8 μM (intermediate between the inner and outer primer concentrations as usual) and at 2.4 μM (in excess of the inner primers). The effect of different loop primer concentrations was assessed by performing the assay on a dilution series of sample 86 in a real time turbidimeter. The concentration of this sample was 11.7 ng μl^{-1} as measured with a NanoDrop Spectrophotometer.

Secondly the assay was re-applied to the total sample set using both 2.4 and 0.8 μM loop primers. For economy, 1 in 10 dilutions of the DNA samples were used to seed the reaction.

The effect of temperature on reaction efficiency and sensitivity was assessed by performing the assay on a dilution series of sample 86 in a real time turbidimeter at 58, 60, 62, 64 and 65 ˚C.

2.5.6 Initial trial of primers for *T. b. gambiense*

A trial using the *TgsGP* LAMP primers was made using *T. b. gambiense TgsGP* PCR positive samples 5 and 17, and non - *T. b. gambiense TgsGP* PCR negative samples 1, 2 and 3. The assay was run at 63 ˚C for 1 hr in a heating block, followed by 2 min at 80 ˚C to halt the reaction. After gel electrophoresis the products were visualised under UV light.

Final concentrations of 2 mM, 4 mM, 6 mM and 8 mM $MgSO_4$ were all attempted, as previous work with novel SRALAMP primers showed that the concentration of $MgSO_4$ affects the LAMP reaction. Otherwise the reaction mix contained: 8 U *Bst* DNA polymerase, 1 X LAMP buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween 20), 0.8 M betaine, 0.2 mM dNTPs, 2 µM FIP and BIP, 0.8 µM LF and LB, 0.2 µM F3 and B3 and H_20 to make the reaction volume up to 25 µl. The reactions were seeded with 1 µl of the DNA sample.

2.5.7 Adaptation of the novel assay for *T. b. gambiense* for use with a real time turbidimeter

This was repeated using the same samples and reaction mix, at 63 ˚C for 90 minutes in the turbidimeter, with a final concentration of 8 mM $MgSO_4$ in the reaction mix. This was trialled with either 0.2 mM or 1.4 mM dNTPs. A reaction termination step was not included when using the real time turbidimeter.

2.5.8 Application of the novel assay for *T. b. gambiense* to the complete sample set using the real time turbidimeter

The LAMP *TgsGP* assay was applied to all 86 samples using a real time turbidimeter. The reaction mix contained 8 U *Bst* DNA polymerase, 1 X LAMP buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$ 2 mM $MgSO_4$, 0.1% Tween 20), plus additional MgSO4 to give a final concentration of 8 mM $MgSO_4$ 0.8 M betaine, 1.4 mM dNTPs, 2 µM FIP and BIP, 0.8 µM LF and LB, 0.2 µM F3 and B3 and H_20 to make the reaction volume up to 25 µl. Temperature was set at 63 ˚C for 90 minutes, and the time to a threshold turbidity (Tt) of 0.1 was noted. Reactions were seeded with 1 µl of the DNA sample.

2.5.9 Application of the novel assay for *T. b. gambiense* to the complete sample set using a heating block

The LAMP *TgsGP* assay was also applied to the 86 samples using a thermocycler to incubate the reaction at 63 ˚C for 60 minutes, followed by a 2 min 80 ˚C termination step. The reactions were then held at 4 ˚C until they were assessed. Assessment was made by optical turbidity assessment, and by gel electrophoresis with subsequent UV illumination for all samples. This full sample set screen was performed three times. For these reactions the reaction mix was the same as with the real time turbidimeter screen described above, except that the reaction buffer contained 0.1% Triton X-100 in place of 0.1% Tween 20. The reactions were seeded with 1 µl of the DNA sample.

2.5.10 Absolute sensitivity of LAMP versus PCR

A two times and a ten times dilution series were made for three *T. b. gambiense* samples (5, 18 and 58) whose DNA concentration was first quantified using a NanoDrop 2000 (Thermo Scientific). One µl of each dilution was used to seed both the *TgsGP* PCR and *TgsGP* LAMP assays. The result was determined both by turbidity in the reaction tube, and by gel electrophoresis and UV visualization.

2.6 Statistical analyses

Two by two contingency tables were made to allow comparisons between any two given tests. From these sensitivity, specificity, positive and negative predictive values and Cohen's kappa statistic were calculated as described below. In order to assist the interpretation of Cohen's kappa statistic the prevalence and bias indices, the 95 % confidence intervals and the maximum attainable kappa were also calculated as described below.

2.6.1 Sensitivity and Specificity

Sensitivity is the proportion of true positives that are detected by the diagnostic test in question. It represents the probability that the test will produce a true positive result when used on an infected population as compared to a reference or 'gold standard'. Conversely the specificity of a test is the proportion of true negatives that are detected by the test, representing the probability that it will produce a true negative result, again as compared to the 'gold standard', when used on a non-infected population. Table 2.9 describes the possible outcomes of any diagnostic test, and the formulae for calculating sensitivity and specificity in comparison to a reference standard.

2.6.2 Positive and Negative Predictive Values

The positive predictive value of a test is the probability that a person is infected when a positive test result is observed whereas the negative predictive value is the probability that a person is not infected when a negative test result is observed. Unlike sensitivity and specificity, the predictive values of a test vary depending upon the prevalence of the condition being tested for in a specific population. Table 2.9 describes the possible outcomes of any diagnostic test, and the formulae for the positive and negative predictive values for any diagnostic test in comparison to a reference standard.

2.6.3 Cohen's kappa

Cohen's kappa statistic (κ) [157] was used to quantify the level of agreement between two tests. This gives a better indication of the concordance between the two techniques than percentage agreement, since it accounts for the agreement expected by chance. The formula for the calculation of kappa is given in Table 2.9. A kappa value of one implies perfect agreement; values less than one imply less than perfect agreement. A negative kappa value indicates that the two tests agree less than would be expected by chance. A rough guide to the interpretation of kappa values has been

given by Altman [199] and has been summarised in Table 2.10 below. However, interpretation of the magnitude of kappa is not straightforward. Benchmarks and thresholds are inevitably arbitrary [200, 201] and the actual magnitude of kappa can be influenced by the prevalence, bias and non independence of ratings [202]. Therefore in this study we follow the recommendations given by Sim and Wright (2005) [202] for the interpretation of kappa: (i) report the bias and prevalence alongside the obtained value of kappa; (ii) relate the magnitude of kappa to the maximum attainable kappa for the contingency table as well as to one in order to provide an indication of the effect of imbalance in the marginal totals; (iii) construct a confidence interval around the obtained value of kappa to reflect sampling error; (iv) be cautious when comparing the magnitude of kappa when prevalence or bias indices differ.

The prevalence index is calculated as the absolute difference between the number of true positive and true negative observations, divided by the total number of observations (Table 2.9) [202]. If the prevalence of a positive rating is either very high, or very low, the prevalence index is high, chance agreement is high and kappa is reduced [200]. The effect of prevalence on kappa is greater for large values of kappa than for small values [203]. It is important to note that the prevalence index itself is affected by both the true prevalence of positives in the population being tested, and by the behaviour of the test or reader.

Bias is the extent to which the two tests, or two raters disagree on the proportion of positive or negative observations. The bias index is calculated as the absolute difference between the number of false positive and false negative observations, divided by the total number of observations (Table 2.9) [202]. When there is a large difference in the number of false positive and false negative observations the bias index is high and kappa is increased. This effect is greater when kappa is small [203]. Therefore, we are faced with two paradoxical situations: (i) kappa is lowered when

the prevalence index is high and (ii) kappa is increased when the bias index is high [204].

The maximum attainable kappa (κ_{max}) is calculated using the same formula as for Cohen's kappa, however, before the calculation is made the marginal probabilities and the cell frequencies are adjusted to obtain the greatest possible agreement. Therefore the difference between the observed kappa and κ_{max} is the unachieved agreement beyond chance within the constraints of the marginal totals.

95 % confidence intervals for kappa were calculated from the contingency table using the GraphPad Quick Calcs online calculator (http://www.graphpad.com/quickcalcs/kappa1.cfm).

Finally kappa assumes that errors are independent [205]. Non- independence increases kappa. Hence, factors affecting the independence of kappa were considered during the interpretation of kappa.

Table 2.9. Possible outcomes of a diagnostic test and associated formulae.

True status	**Test status**		**Total**
	Positive	**Negative**	
Positive	True Positive (TP)	False Negative (FN)	TP+FN
Negative	False Positive (FP)	True Negative (TN)	FP+TN
Total	TP+FP	FN+TN	TP+FN+FP+TN
Sensitivity = TP / (TP + FN)			
Specificity = TN / (TN + FP)			
Positive Predictive Value = TP / (TP + FP)			
Negative Predictive Value = TN / (TN + FN)			
κ = (Total number of agreements-Expected number of agreements)/(Total number of observations-Expected number of agreements)			
Prevalence index = (| TP – TN |)/ (TP+FN+FP+TN)			
Bias index = (| FP – FN |)/ (TP+FN+FP+TN)			

Table 2.10. The interpretation of Cohen's kappa statistic [199]

Kappa value	Level of agreement
<0.00	Less than would be expected by chance
0.00 – 0.20	Poor agreement
>0.20 – 0.40	Fair agreement
>0.40 – 0.60	Moderate agreement
>0.60 – 0.80	Good agreement
>0.80 – 1.00	Very good agreement

2.7 Multi observer study

Thirty-three observers each scored all 60 samples as positive or negative by comparison with a positive and negative control, after training on a small batch of eight samples. The participants were not chosen according to any specific criteria but were asked about their work background, previous experience of colour change assays and their impressions of these tests in light of their previous experience. Observers were asked to rank how easy they found differentiation of the colour differences (very easy, quite easy, quite difficult or very difficult), and how many of the samples were easy to rate as positive or negative (all, most, some, very few or none) for each method. They were also asked which colour change method they found easiest to use. Finally they were given the opportunity to make any general comments.

3 Chapter 3. Detection of human infective trypanosomes in the zoonotic reservoir using LAMP

3.1 Introduction

Mathematical modelling has clearly demonstrated that control of *T. b. rhodesiense* in the animal reservoir, in addition to early case detection and treatment in humans, is critical for the control of Rhodesian sleeping sickness [34]. However, identification of *T. b. rhodesiense* in domestic livestock is difficult for two reasons: (i) *T. brucei* s.l. infection in domestic livestock is not usually clinically apparent [206] and (ii) microscopically *T. b. rhodesiense* cannot be differentiated from non-human-infective *T. b. brucei,* which may be circulating among the same reservoir host species. Morphological differentiation of *T. brucei* from *T. congolense* and *T. vivax* also remains difficult, insensitive and error-prone [207, 208].

Following the discovery of the *SRA* gene (which uniquely identifies *T. b. rhodesiense* at the DNA level) it has been possible to detect and quantify human-infective parasites in the unaffected livestock reservoir. In areas endemic for human sleeping sickness one in three *T. brucei* s.l. infections in cattle can be identified as *T. b. rhodesiense* [209]. Assuming this ratio is constant it should now therefore be possible to indirectly quantify circulating human parasites, using a generic *T. brucei* s.l. diagnostic. To this end, molecular techniques have come to the forefront of trypanosome detection technologies. Early work made use of DNA probes for the detection of *Trypanozoons*, targeting a 177 bp satellite repeat sequence [193] and the *Trypanozoon* ingi element [210, 211]. The next significant advance came with the development of PCR, which was first applied to trypanosomes in 1989 by Moser [107] whose designed primers to the same 177 bp satellite repeat sequence described above (of which there are 10000 copies per haploid genome). Twenty years on this *Trypanozoon* specific PCR remains the method of choice for the molecular detection of these parasites. It is sensitive to as little as 0.01 pg of DNA; equivalent to one

tenth of a trypanosome and has been successfully applied in several large-scale epidemiological studies [32, 195] that have screened DNA from cattle blood samples collected onto Whatman FTA cards. These FTA cards provide an ideal matrix for sample collection and transport from remote, ill-equipped field sites, as well as for long-term sample storage without the requirement for refrigeration or any other special storage facilities. In addition, two different loop-mediated isothermal amplification (LAMP) assays have recently been developed for the detection of *Trypanozoon* DNA: one targeting the single copy *PfrA* gene [140] and the other targeting the multi-copy RIME element [121].

RIME is a mobile genetic element first described in a ribosomal DNA clone from *T. brucei* in 1984 [212]. Mobile genetic elements can be classified according to their transposition mechanism, sequence structure and insertion site specificity. RIME is a random, non-long terminal repeat, retrotransposon; thus it moves randomly through the trypanosome genome via an RNA intermediate. It is the most common mobile genetic element in trypanosomes making up approximately 5 % of the trypanosome genome and being present at about 400 copies [210, 213, 214]. Although it lacks strict insertion site specificity, it is clustered, particularly within the rRNA genes and the telomeres of chromosome one [215-217]. Before development of the LAMP RIME assay, the RIME element had been exploited for strain typing of *T. brucei* s.l. via an MGE-PCR assay [218-220]. Mobile genetic elements are often well suited for strain typing because their location and length may vary between strains.

The LAMP RIME primers were designed by Njiru *et al.* [121], who used RIME sequences from *T. b. brucei* (accession number EF567426), *T. b. rhodesiense* (accession number EF567426) and *T. evansi* (accession number EF567425) as their templates. These were sequences were generated from PCR products amplified using primers designed from the original RIME sequence (accession number K01801; [212]).

The *PfrA* (paraflagellar rod protein A) gene (accession number X14819) of *T. brucei* was first identified and sequenced by Schlaeppi *et al.* in 1989 [221]. At that time the authors believed that *PfrA* and *PfrB* were two identical, tandemly linked genes, found at a single locus in the trypanosome genome, which both coded for a 600 amino acid protein via distinct mRNAs. The paraflagellar rod (PFR) was first identified in 1962 [222]. It is a major component of kinetoplastid and euglenoid flagellae alongside the microtubular axoneme in these flagellate organisms. Early studies identified two major protein components of the paraflagellar rod, PFR1 and PFR2. More recent work has identified over 40 additional proteins associated with the PFR [223]. Schlaeppi *et al*. suggested that PFR1 and PFR2 were encoded by one gene with the observed differences being due to protein folding [221]. However genome sequencing of *T. brucei*, *Trypanosoma cruzi* and *Leishmania major* identified distinct (though related) genes encoding PFR1 and PFR2 which are present in separate tandem arrays and have arisen via a single gene duplication event [215, 224, 225]. Note that the *Pfr1* and *Pfr2* genes were sequenced after the development of the LAMP *PfrA* assay. They are available via GenBank under accession numbers XM_838928 and XM_842234 respectively.

In this study the LAMP *PfrA* [140] and LAMP RIME [121] assays were compared to the well established TBR PCR [107] for the detection of members of the subgenus *Trypanozoon* in cattle blood samples collected onto Whatman FTA cards. This study was performed in order to determine whether LAMP might be a useful and simpler alternative to PCR as part of remote epidemiological surveillance studies.

3.2 Aims

Initially there were two main aims to this study:

1. To compare the sensitivity of LAMP *PfrA*, LAMP RIME and TBR PCR assays for detecting members of the subgenus *Trypanozoon* from cattle blood samples collected as part of ongoing epidemiological surveillance.
2. To compare the cost of LAMP *PfrA*, LAMP RIME and TBR PCR assays for detecting members of the subgenus *Trypanozoon*.

As analysis of results arising from this section work began it became apparent that LAMP RIME detected an excess of *Trypanozoon* positive samples. The further aims of the study were then defined as follows:

3. To investigate the possibility of LAMP positive reactions from non-*Trypanozoon* DNA
    a. Using *Trypanosoma congolense* and *Trypanosoma vivax* DNA
    b. Using cattle blood samples with a high *T. congolense* infection rate
    c. Using host genomic DNA
    d. By sequence analysis of the primer binding sites
4. To design an improved LAMP assay for detecting members of the subgenus *Trypanozoon*

3.3 Study outline

Firstly, blood samples from Ugandan cattle were used for the detection study which compared LAMP RIME [121], LAMP *PfrA* [140] and TBR PCR [107]. Prior to this study no other characterisation of these samples had taken place, nor did they come from animals identified as cases or controls. Therefore, this molecular data could only be analysed in isolation, without reference to any external gold standard. Initially, the results from the LAMP *PfrA* and LAMP RIME assays were compared to the results from the TBR assay, which is considered as the current reference standard for the molecular diagnosis of *Trypanozoon* parasites. Sensitivity, specificity, negative and positive predictive values, Cohen's kappa statistic with 95

% confidence intervals, prevalence and bias indices and the maximum attributable kappa were calculated. Next, an extended reference standard was defined that incorporated the results from all three assays. Samples that generated a positive signal using any of the three assays were considered positive. Sensitivity measures were calculated for each assay against this reference standard. Sensitivity was also calculated for the use of two assays in parallel, where the combination was taken as positive if either one, or both, gave a positive signal. The chi-squared test was used to analyse whether the differences seen in the sensitivity arising with the different molecular approaches were statistically significant.

Secondly, the costs of the reagents required for the three assays were compared. The present cost (at the time of writing) for each reagent was noted from the appropriate supplier website or from our previous order information where pricing was not publicly available. The quantity of each reagent required for 100 reactions was calculated to determine the total cost for 100 reactions of each assay. The cost calculations are shown in Appendix 3.

Having observed an excess of LAMP RIME positive reactions, in comparison to the TBR PCR, with the Ugandan cattle blood samples, it was deemed necessary to revisit the LAMP *PfrA* and LAMP RIME assays. So, thirdly, the specificity of these LAMP assays was scrutinized using: (i) *T. congolense* (Fly148) and *T. vivax* (TREU 1722) DNA extracted previously from procyclic trypanosome cultures using the Qiagen DNAeasy Blood and Tissue Kit (Qiagen,UK); (ii) 105 Zambian cattle blood samples with a high known prevalence of *T. congolense* savannah infection and (iii) commercially available human and cattle genomic DNA.

For (i) both the ITS1 rDNA PCR and the TBR PCR were performed once on a tenfold dilution series of each DNA, using 1 µl template DNA. The LAMP RIME and LAMP *PfrA* assays were performed in triplicate on the tenfold dilution series of

DNA, and then an additional ten times on the strongest concentration. For (ii) the LAMP RIME assay was applied to all 105 samples. For (iii) eight negative control samples (seeded with H_20) and one positive *Trypanozoon* DNA sample were ran alongside LAMP RIME and LAMP *PfrA*.

Fourthly a BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) [226] search was performed for EF567424, EF567425, EF567426 and X14819 (LAMP RIME and LAMP *PfrA* primer target sequences) to identify similar non-*Trypanozoon* sequences. Hits were considered according to the following criteria: (i) inclusion of the full LAMP primer binding region; (ii) percentage sequence identity. When the entire primer binding region was included and sequence identity was high the precise primer binding locations were mapped within the BLAST hit.

Finally, attempts were made to design a *Trypanozoon* specific LAMP assay targeting the same 177 bp sequence used for TBR PCR primer design using the Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html).

3.4 Results

3.4.1 Comparative detection study

The two LAMP assays (RIME and *PfrA*) and the TBR PCR were applied to 428 cattle blood samples. Seventy two samples were TBR PCR positive, 23 samples were LAMP *PfrA* positive and 145 samples were LAMP RIME positive. In total 189 samples were positive by one or more of the assays.

3.4.1.1 Comparison to current reference standard

The TBR PCR is considered to be the gold standard for the molecular diagnosis of *Trypanozoon* parasites and is routinely applied to cattle blood samples collected onto Whatman FTA cards to screen for *T. brucei* s.l.. Therefore, data analysis began with the comparison of LAMP *PfrA* and LAMP RIME to TBR PCR.

LAMP *PfrA* showed less agreement than would be expected by chance alone with TBR PCR (κ = - 0.02; 95 % CI -0.209 – 0.169) while LAMP RIME showed fair agreement with the TBR PCR (κ = 0.233; 95 % CI 0.123 – 0.344). With LAMP *PfrA* the majority of the discordant results were false negatives, while for LAMP RIME they were false positive. The contingency table and summary statistics are shown below (Tables 3.1 and 3.2).

These comparisons are also summarised graphically below (Figure 3.1).

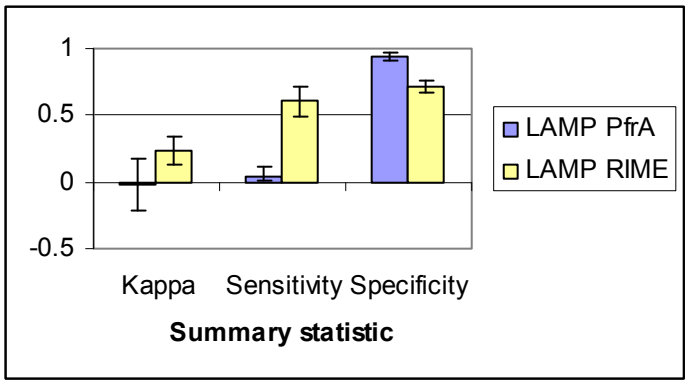
Table 3.1. Contingency tables for the comparison of the LAMP reactions with the current standard TBR PCR

	LAMP *PfrA*			**LAMP RIME**		
TBR PCR	+	-	**Total**	+	-	**Total**
+	3	69	72	44	28	72
-	20	336	356	101	255	356
Total	23	405	428	145	283	428

Table 3.2. Comparative summary statistics when the two LAMP assays were compared to the TBR PCR assay

	LAMP *PfrA*	**LAMP RIME**
Kappa	-0.02	0.233
(95 % CI)	(-0.209, 0.169)	(0.123, 0.344)
Sensitivity	4.17 %	61.11 %
(95 % CI)	(0.94 – 12.03 %)	(49.55 – 71.55 %)
Specificity	94.38 %	71.63 %
(95 % CI)	(91.44 – 96.38 %)	(66.73 – 76.07 %)
N P V	82.96 %	90.11 %
(95 % CI)	(78.98 – 86.32 %)	(86.03 – 93.11 %)
P P V	13.04 %	30.34 %
(95 % CI)	(3.69 – 32.97 %)	(23.43 – 38.27 %)

Figure 3.1. Kappa, sensitivity and specificity estimates for LAMP *PfrA* and LAMP RIME compared to TBR PCR, with 95 % confidence intervals



To aid interpretation of kappa the prevalence and bias indices were calculated, as well as the maximum attributable kappa, for the agreement between LAMP *PfrA* and TBR PCR, as well as for LAMP RIME and TBR PCR (Table 3.3). The maximum attributable kappa fell well short of 1 for both comparisons, and the prevalence index was high. Therefore, kappa may be artificially deflated by the skew in the data towards samples that are negative by both tests being compared.

Table 3.3. Prevalence and bias indices, and the maximum attributable kappa for LAMP *PfrA* and LAMP RIME versus TBR PCR

	LAMP *PfrA*	**LAMP RIME**
κ_{max}	0.438	0.566
Prevalence index	0.778	0.493
Bias index	0.114	0.171

3.4.1.2 Comparison to the extended reference standard

According to the results described in Table 3.1 above LAMP RIME signals were detected in 101/356 TBR negative samples (28.37 %; 95 % CI 23.93 – 33.27 %). LAMP *PfrA* signals were also detected in 5.62 % TBR negative samples (n = 20/356;

95 % CI 3.62 – 8.56 %). Therefore an extended reference method was defined: a sample was considered to contain *Trypanozoon* DNA if it was positive by any one or more of the three assays. This standard assumes that all three targets are perfectly specific, and that disagreements arise because they target different regions of the trypanosome genome.

TBR PCR identified 38.10 % (n = 72/189; 95 % CI 31.47 – 45.19 %) of the samples defined as positive in this study. This was more sensitive than LAMP *PfrA* which detected only 12.17 % (n = 23/189; 95 % CI 8.19 – 17.66 %). However, LAMP RIME assay was more sensitive than the PCR, detecting 76.72 % (n = 145/189; 95 % CI 70.18 – 82.20 %) of the positive samples.

When the results from more than one assay were combined the sensitivity was improved. The best combination, and most sensitive measure overall, was seen when the results from the PCR and LAMP for RIME assays were combined. Here, 91.53 % (n = 173/189; 95 % CI 86.61 – 94.83 %) of positive samples were identified.

The χ^2 results indicate a strong association between the type of assay, or combination of assay results, and the detection sensitivity. Table 3.4 displays the sensitivity seen with each approach, alongside the χ^2 statistics.

Finally, the less sensitive assays detected some positive samples not identified by the more sensitive assays. Therefore a more sensitive assay cannot be relied upon to detect *Trypanozoon* DNA in all the samples detected by a less sensitive assay.

Table 3.4. Sensitivity of each method relative to the total number of positive samples identified in the study

Method	Sensitivity (95 % CI)	χ^2
Single assay		
PCR	38.10 % (31.47 – 45.19 %)	χ^2= 94.225 df =2 p<0.0001
LAMP for *PfrA*	12.2% (8.19 – 17.66 %)	
LAMP for RIME	76.7% (70.18 – 82.20 %)	
Two assays		
PCR plus LAMP for *PfrA*	48.68 % (41.65 – 55.76 %)	χ^2= 26.915 df =2 p<0.0001
PCR plus LAMP for RIME	91.53 % (86.61- 94.83 %)	
LAMP for RIME plus LAMP for *PfrA*	85.19 % (79.37 – 89.59 %)	

3.4.1.3 Cost comparison

The TBR PCR assay was the cheapest, in terms of reagents alone, costing £21.57 for 100 reactions. LAMP RIME, which costs £31.09 for the reagents required for 100 reactions, is cheaper than LAMP *PfrA*, which costs £42.40 for the reagents required for 100 reactions. LAMP *PfrA* is more expensive than LAMP RIME owing to the higher concentration of dNTPs required, and the addition of $MgSO_4$ to the reaction.

3.4.2 Non - specific LAMP RIME reactions

Above, the sensitivity of single and parallel testing with TBR PCR, LAMP *PfrA* and LAMP RIME were compared to an extended reference standard. As noted, the validity of these comparisons depends on the specificity of these molecular assays. However, it is possible that the LAMP positive signals detected in TBR PCR negative samples were false positives. To investigate this possibility LAMP RIME and LAMP *PfrA* were applied to: (i) *T. congolense* and *T. vivax* DNA; (ii) Zambian cattle blood samples with a high known prevalence of *T. congolense* savannah infection and (iii) commercially available human and cattle genomic DNA.

3.4.2.1 With T. congolense and T. vivax DNA

The identity of the *T. congolense* (Fly 148) and *T. vivax* (TREU 1722) DNA samples were confirmed by the ITS1 rRNA PCR. Neither of these DNA samples were *Trypanozoon* positive, either according to the ITS1 PCR or the TBR PCR. When the LAMP RIME and *PfrA* assays were applied in triplicate to each dilution series, one LAMP RIME positive reaction was observed for *T. congolense*, and 15 for *T. vivax* (Table 3.5). Thirteen of the *T. vivax* LAMP RIME positives were observed for dilutions from which no trypanosome DNA was detected by the ITS1 rDNA PCR. No LAMP *PfrA* positives were observed.

Table 3.5. LAMP and PCR performed on dilution series of *T. congolense* and *T. vivax* DNA

	T. congolense				***T. vivax***			
	TBR	**ITS1 rDNA**	**Number of positive LAMP signals (n = 3)**		**TBR**	**ITS1 rDNA**	**Number of positive LAMP signals (n = 3)**	
			RIME	***PfrA***			**RIME**	***PfrA***
1 X	-	700 bp	1	0	-	250 bp	2	0
1 X 10^{-1}	-	700 bp	0	0	-	-	1	0
1 X 10^{-2}	-	-	0	0	-	-	3	0
1 X 10^{-3}	-	-	0	0	-	-	3	0
1 X 10^{-4}	-	-	0	0	-	-	1	0
1 X 10^{-5}	-	-	0	0	-	-	2	0
1 X 10^{-6}	-	-	0	0	-	-	3	0

LAMP RIME and *PfrA* assays were then performed ten times each on the most concentrated *T. congolense* and *T. vivax* DNA. Again, all LAMP *PfrA* reactions were negative. However two LAMP RIME positives were observed; one with *T. congolense* and one with *T. vivax*. These results are shown in Table 3.6.

Table 3.6. LAMP and PCR performed on *T. congolense* and *T. vivax* DNA

	TBR	ITS1 rDNA	Number of positive LAMP signals (n = 10)	
			RIME	*PfrA*
1 X *T. congolense*	-	700 bp	1	0
1 X *T. vivax*	-	250 bp	1	0

3.4.2.2 With Zambian cattle blood samples

The sample set contained 105 samples, of which 70 had previously been identified as PCR positive for *T. congolense* savannah, ten as PCR positive for the sub-genus *Trypanozoon*, and one as PCR positive for *T. vivax*. This includes five *T. brucei – T. congolense* savannah mixed infections, and one *T. vivax – T. congolense* savannah mixed infections. Overall, in this study, eight samples were LAMP RIME positive.

The distribution of these LAMP RIME positives is shown in Table 3.7 below.

Table 3.7. LAMP RIME positive samples among the Zambian cattle blood samples

PCR identification	**LAMP RIME**		
	Positive (%)	**Negative (%)**	**Total**
T. brucei	0	5	5
T. congolense Savannah	3	61	64
T. brucei & *T. congolense*	1	4	5
T. vivax & *T. congolense*	1	0	1
No PCR positive ID	3	27	30
Total	**8 (7.62%)**	**97 (92.38 %)**	**105**
All trypanosome positive	5 (6.67%)	70 (93.33 %)	75
All trypanosome positive, *Trypanozoon* negative	4 (6.15%)	61 (93.85%)	65

LAMP RIME identified *Trypanozoon* positive samples with 10.00 % sensitivity (n = 1/10; 95 % CI 0.00 – 42.60 %) and 92.63 % specificity (n = 88/95; 95 % CI 85.33 – 96.62 %). The agreement between LAMP RIME and TBR PCR is poor [199] (κ = 0.029; 95 % CI -0.409 – 0.467). Tables 3.8 and 3.9 summarise the agreement and agreement statistics for the comparison between LAMP RIME and TBR PCR.

Table 3.8. Contingency table for the agreement between LAMP RIME and TBR PCR

		LAMP RIME		
		+	-	**Total**
TBR PCR	+	1	9	10
	-	7	88	95
	Total	8	97	105

Table 3.9. Kappa statistic for the agreement between LAMP RIME and TBR PCR

Kappa (95 % CI)	0.029(-0.409, 0.467)
Prevalence index	0.829
Bias index	0.019
κ_{max}	0.879

Some (n= 4/65; 6.15 %) LAMP RIME positive reactions were seen among *T. congolense* positive – *Trypanozoon* negative samples. However, widespread false positives were not seen. LAMP RIME detected only a small proportion (n = 5/70; 7.14 %) of *T. congolense* savannah PCR positive samples and agreement between LAMP RIME and the *T. congolense* savannah PCR is less than would be expected by chance [199] (κ = -0.01; 95% CI -0.152 – 0.133). Tables 3.10 and 3.11 summarise the agreement between LAMP RIME and the PCR for *T. congolense* savannah.

Table 3.10. Contingency table for the agreement between LAMP RIME and TCS PCR

		LAMP RIME		
		+	-	**Total**
TCS PCR	+	5	65	70
	-	3	32	35
	Total	8	97	105

Table 3.11. Kappa statistic and sensitivity for the agreement between LAMP RIME and TCS PCR

Kappa (95 % CI)	-0.01 (-0.152 – 0.133)
Prevalence index	0.257
Bias index	0.590
κ_{max}	0.08
Sensitivity (95 % CI)	7.14 % (2.72 – 16.02 %)

3.4.2.3 With commercially available human and cattle genomic DNA

Positive reactions were observed for LAMP *PfrA* and LAMP RIME assays when the most concentrated host DNA was used (12.5 ng human placental DNA, or 50 ng calf thymus DNA added to a single 25 µl reaction). No further LAMP *PfrA* positive results were seen. Two additional LAMP RIME positives were observed in an uneven distribution across the human DNA dilution series and four additional LAMP RIME positives were observed in an uneven distribution across the cattle DNA dilution series (Table 3.12).

Table 3.12. LAMP *PfrA* and RIME results performed on dilution series of human and cattle DNA

	Calf thymus DNA		Human placental DNA	
Dilution	**LAMP *PfrA***	**LAMP RIME**	**LAMP *PfrA***	**LAMP RIME**
1 X	+	+	+	+
1 X 10^{-1}	-	+	-	-
1 X 10^{-2}	-	-	-	+
1 X 10^{-3}	-	-	-	-
1 X 10^{-4}	-	+	-	-
1 X 10^{-5}	-	+	-	+
1 X 10^{-6}	-	+	-	-
1 X 10^{-7}	-	-	-	-

3.4.3 Sequence analysis

In order to take the investigation of LAMP *PfrA* and LAMP RIME specificity a step further a BLAST search was made for the *Trypanozoon* sequences which were used for LAMP primer design. (For LAMP RIME primer design these were EF567424 (*T. b. rhodesiense*), EF567425 (*T. evansi*) and EF567426 (*T. b. brucei*); for LAMP it was X14819). Matches to the LAMP RIME primer template sequences were seen in *Trypanosoma cruzi* and *Schistosoma mansoni*. None of these *T. cruzi* sequences contained the entire LAMP primer binding region, therefore non-specific binding to this parasite was discounted. However, potential LAMP RIME primer binding sites were identified within *S. mansoni*.

Out of the five *Schistosoma mansoni* hits, four were located in a genome sequence supercontig (Smp_scaff010453, accession number: FN367744.1) and the fifth was

located in a hypothetical protein mRNA (Smp_186500, accession number XM_002570283.1). One of these fragments included the entire LAMP primer binding sequence and has 99 % sequence identity with EF567424 and EF567426. The LAMP RIME primer binding sites could be located in this fragment with only one 1 bp sequence mismatch observed in the F1c primer. These five hits, and the LAMP RIME primer binding sites in *Schistosoma mansoni* FN367744.1 are shown in Appendix 4.

Several non-*Trypanozoon* sequences were identified by BLAST performed on X14819, the sequence used for LAMP *PfrA* primer design (Table 3.13). However, of those that contained the LAMP *PfrA* primer binding region none had more than 85 % sequence identity with X14819. Therefore it was considered unlikely that LAMP *PfrA* primers would initiate false LAMP amplification for these species.

Table 3.13. BLAST hits against the sequence used for LAMP *PfrA* primer design

Non- *Trypanozoon* organism	**Number of hits by BLAST**
Trypanosoma cruzi	18
Leishmania	22
Crithidia fasciculata	2
Herpatomonas megaseliae	2
Phacus smulkowskianus	2
Total	**46**

3.4.4 Designing novel *Trypanozoon* specific LAMP primers for the 177 bp TBR PCR target sequence

It was not possible to design a set of LAMP primers for the same sequence targeted by the *Trypanozoon* specific TBR PCR. LAMP primer design software requires a minimum sequence of 200 bp while the TBR PCR target sequence is only 177 bp in length.

3.5 Discussion

This is the first reported use of the LAMP methodology for the detection of *Trypanozoon* from cattle blood samples. More than 400 cattle blood samples collected onto Whatman FTA cards were used to compare a well established PCR methodology against two published LAMP reactions – LAMP RIME and LAMP *PfrA*.

The LAMP RIME reaction detected far more '*Trypanozoon* positive' samples than the well established PCR protocol. However, LAMP *PfrA* detected far fewer

'*Trypanozoon* positive' samples than the well established PCR protocol. The detection sensitivity was significantly improved when results from more than one assay were combined – a parallel testing approach. LAMP RIME with the PCR was the most sensitive combination and most sensitive measure overall.

The different sensitivities may be attributed to the different DNA targets of the three assays, and the different reaction mechanisms by which PCR and LAMP proceed. The PCR primers target a 177 bp repeat of which there are 10000 copies per haploid genome [107, 193, 194]. RIME is a mobile genetic element present at up to 400 copies in the *T. brucei* genome [219]. Thus, though less prolific than the PCR target, the LAMP for RIME assay also uses a multi-copy sequence. In contrast the LAMP for *PfrA* primers target the single copy *PfrA* gene [140]. The LAMP reaction proceeds by a very different mechanism to traditional PCR [123]. It can generate a much higher amplicon concentration in a shorter time frame and is therefore typically more sensitive than PCR. The reaction is particularly efficient when loop primers are included [125], as is the case for LAMP for RIME. Indeed, the absolute sensitivities for these assays reported by the original authors match the hierarchy of relative sensitivities reported in this study. The LAMP for RIME assay is reported to be 100 times more sensitive than the PCR with a detection limit of 0.0001 pg of DNA, whereas the LAMP for *PfrA* requires a minimum of 1 pg of DNA, equivalent to 10 trypanosomes ml^{-1}.

These different approaches also differ in cost. Considering reagents alone the cost per 100 reactions is cheapest for the PCR assay (£21.57 for 100 reactions) and most expensive for the LAMP *PfrA* assay (£42.40 for 100 reactions). Therefore, while LAMP RIME is apparently more sensitive than the equivalent PCR, it is more expensive. However, if the application of LAMP continues to grow (see Chapter 1) it is likely that the price of *Bst* DNA polymerase will fall. The cost of this polymerase accounts for more than half the cost of the LAMP reaction (Appendix 3). According to the Bioline customer services helpline the cost of *Taq* polymerase (catalogue

number 21060) has been unchanged for the last five years at £350. Using the retail price indices (RPI) for July 2005 (758.2) and July 2010 (884.1) it is possible to calculate that £350 in 2005 is equivalent to £408.18 today. In effect therefore the price of *Taq* has fallen, as one might expect *Bst* to do also. A thermal cycler, necessary for PCR reactions, is an extra cost compared to the water bath required for LAMP assays. Thermal cyclers range in price according to functional specification and supplier. A quick web search found that one UK supplier quotes £1965.00 for the cheapest available thermal cycler (25 Well Thermal Cycler, TC-300; catalogue number MB0530; www.appletonwoods.co.uk) compared to £242.05 for the least expensive dry heating block (Labnet Accublock, single dry block heater with 20 wells; catalogue number HE1 501; www.appletonwoods.co.uk). Therefore the additional cost of the PCR machine (£1723) is equivalent to the extra cost for 18000 reactions LAMP RIME - after 18000 samples have been processed the cost of the thermal cycler is offset and PCR becomes the cheaper option.

Given the excess of LAMP RIME positive results over and above the detection of TBR PCR positives, sensitivity differences might not be the only difference between the assays. It was important to check the specificity of the LAMP reaction. Non-specific LAMP RIME amplification with other bovine infective trypanosomes, as well as non-specific amplification from host DNA were investigated. The results were not encouraging. LAMP RIME amplified *Trypanosoma congolense* and *Trypanosoma vivax* DNA, as well as human and cattle genomic DNA. LAMP *PfrA* positive reactions were also seen with most concentrated host genomic DNA samples. One can consider what this might mean for user contamination in the laboratory. Recall that a tenfold dilution series was made and that the most human DNA added to the LAMP assays (with the 1 X dilution) was 12.5 ng. This is more than 1000 times the DNA content of a human cell (estimated at 7.2 -7.5 pg [227]). However, LAMP RIME positive amplification was seen with human DNA equivalent to less than that seen in a single human cell. Therefore the technician could easily contaminate the LAMP assay via skin shedding or during handling.

What is more, small amounts of human DNA are very likely to contaminate the FTA card, unless gloves are strictly worn. In addition LAMP RIME positive reactions were seen with *T. congolense* savannah PCR positive-*Trypanozoon* PCR negative cattle blood samples. Notably, the results presented in this chapter contradict the original study, in which LAMP RIME specificity was assessed against DNA samples (approx 1 ng) human, tsetse, bovine, camel, *Plasmodium falciparum*, *T. congolense* savannah, *T. congolense* kilifi, *T. congolense* forest, *T. simiae*, *T. simiae* tsavo, *T. godfreyi*, *T. vivax* and *T. lewisi* and in which no positive reactions were reported for any of these samples [121].

Finally, the BLAST search results give rise to real concern that LAMP RIME could amplify DNA from *Schistosoma mansoni*. *S. mansoni* is one of five main species of trematode worm that cause Schistosomiasis – a chronic parasitic disease infecting 207 million people worldwide, 85 % of whom live in sub-Saharan Africa (http://www.who.int/mediacentre/factsheets/fs115/en/index.html). For example, in Uganda, in 2003, 20.4 % of the population were estimated as infected [228]. Although the focus of this study is the use of LAMP RIME for the detection of *Trypanozoon*s in cattle, others are evaluating the use of LAMP RIME for the detection of *Trypanozoon*s in humans [229]. Cross reactivity of the LAMP RIME assay with *S. mansoni* would seriously undermine its specificity in its target human population.

The LAMP *PfrA* assay was published in 2003. In the seven years since its publication it has been applied in only three additional research publications: (i) a study in which LAMP *PfrA* was compared to alternative diagnostic approaches for *Trypanosoma evansi* (Surra) in pigs [139]; (ii) a study assessing the thermostability of LAMP reagents and its amplification efficiency on crude trypanosome DNA templates [230] and (iii) a comparison of the analytical sensitivity of LAMP RIME and LAMP *PfrA* [121]. Firstly, LAMP *PfrA* specifically detected *T. evansi* in experimentally infected pigs, with high sensitivity, which exceeded that of PCR and

microscopy, and was second only to mouse inoculation [139]. Secondly, storage of LAMP reagents at 25 ˚C, 37 ˚C or -20 ˚C did not affect the detection sensitivity of LAMP *PfrA* and, although the detection sensitivity was poor when fresh blood was used to directly seed the LAMP *PfrA* reaction, hemolysed blood provided a suitable template for LAMP amplification [230]. Thirdly, LAMP *PfrA* required a 50 fold higher concentration of *Trypanozoon* DNA for successful amplification as compared to LAMP RIME [121].

The LAMP RIME assay was published more recently, in 2008, having been developed by FIND (Foundation for Innovative New Diagnostics) with its academic partners at Murdoch and Obihiro Universities [115]. By selecting a gene with a high copy number, they hoped to improve the analytical sensitivity of *Trypanozoon* detection, in comparison to the LAMP *PfrA* assay. They succeeded. FIND continue to advocate the use of LAMP RIME as a potential new diagnostic for HAT (http://www.finddiagnostics.org/programs/hat/find_activities/molecular_diagnosis.html). However, this study suggests that this improvement in analytical sensitvity brings an unacceptable cost in terms of reduced specificity.

Recently, the LAMP RIME and a LAMP assay specific for the detection of *T. b. rhodesiense* (LAMP *SRA* [122]) have been compared to an *SRA* PCR [231] protocol also using human patient blood spotted onto FTA cards [229]. There are several key differences between the methodology and key findings of this paper, and the study reported here, which are worthy of note. Firstly, the samples were collected from human patients, confirmed as trypanosome positive by microscopy. Hence specificity could not be assessed, rather the study focused on sensitivity. Second, LAMP RIME was not compared to TBR PCR, which detects all members of the subgenus *Trypanozoon* by targeting the multicopy 177 bp satellite repeat sequence. Rather, both LAMP reactions were compared to an *SRA* PCR, which has a single copy gene target. The study demonstrated that LAMP RIME and LAMP *SRA* are both more sensitive than the *SRA* PCR protocol used, and, further, that LAMP RIME and

LAMP *SRA* show very good agreement with one another (κ = 0.85, 95% CI 0.64-1). As the authors themselves note, a case control study is a key next step, in which LAMP RIME and LAMP *SRA* are applied to trypanosome negative as well as trypanosome positive blood samples. However, the results shown in this chapter suggest that as a research community we need to be much more confident about the specificity of the RIME assay before such steps forward are taken. Strategies to investigate LAMP RIME specificity further are urgently required, and will be discussed in Chapter 9. Finally, as noted elsewhere [232], this study highlights the variety of PCR protocols in circulation, and the lack of a unified standard against which LAMP might be compared.

Since its publication in 1989 the TBR PCR has enjoyed widespread use. It has been applied:

(i) for the detection and identification of trypanosomes in tsetse [39, 233-235]),

(ii) to incriminate animal reservoirs of *T. b. gambiense* e.g. [236] and *T. b. rhodesiense* e.g. [33],

(iii) for post treatment follow up of *T. brucei* in cattle [237, 238],

(iv) during investigation of fatal post treatment reactive encephalopathies in human patients [239],

(v) as a diagnostic for *T. b. gambiense* sleeping sickness, particularly as a follow up to CATT where there is discrepancy between parasitological detection and serology [86, 91, 92, 240],

(vi) to detect *Trypanosoma evansi* in camels [241], other mammals [242], and even an atypical human infection [23],

(vii) to detect *Trypanosoma equiperdum* in horses [243],

(viii) as a putative stage determining diagnostic in sleeping sickness patients [244] and (viiii) for diagnosis from archived human blood slides [39].

This list is not exhaustive, yet it highlights the utility of a molecular diagnostic that is capable of sensitive and specific detection of the *Trypanozoon*s. In addition the TBR PCR is routinely used in our laboratory to monitor the prevalence of *T. brucei* s.l. as part of the Stamp Out Sleeping Sickness Campaign (www.stampoutsleepingsickness.com). The World Organisation for Animal Health (OIE) do not prescribe any particular test for tsetse transmitted animal trypanosomiases for the purposes of international trade.

Here we ask: does LAMP offer a useful alternative to TBR PCR for remote epidemiological surveillance of cattle blood samples collected onto Whatman FTA cards? In this context the thermostability of LAMP reagents is largely irrelevant.

At present we have serious concerns about both *Trypanozoon* LAMP reactions, particularly the sensitivity of LAMP *PfrA* and the specificity of LAMP RIME. Further, both LAMP reactions are more expensive than TBR PCR (for reagents alone).

Unfortunately, attempts here to design LAMP primers to the sequence targeted by the TBR PCR were unsuccessful. Should an improved LAMP assay be developed, whose sensitivity and specificity is comparable, or even better than the TBR PCR, LAMP might be useful for more local monitoring of disease prevalence in cattle. LAMP ought to be a better suited technology for use in a less well resourced laboratory. Heating blocks are less expensive than PCR machines, or a water bath could be used and end product visualization can be achieved by addition of a cheap colour change reagent such as hydroxynaphthol blue (see also Chapter 8).

If LAMP does not currently provide a suitable alternative to PCR for surveillance of *T. brucei* s.l. in cattle, might it be a useful tool for human diagnoses? Here, cross reactivity with *T. congolense* and *T. vivax* would be less relevant, since these species

are not typically human-infective. However at least one mixed *T. brucei – T. congolense* human infection has been reported [40]. Further, it is plausible that *T. congolense* or *T. vixax* DNA might persist in the human blood stream for long enough to be detected after lysis of these parasites. The apparent amplification of host DNA is also important. According to the Qiagen DNeasy Blood and Tissue Kit (Qiagen, UK) 3-6 µl DNA can be purified from 100 µl mammalian blood (approximately equivalent to the volume of blood spotted onto an FTA card) into 100 µl elution buffer, to give a DNA concentration of 0.03 – 0.06 µg μl^{-1}. By comparison our starting concentrations of human and cattle genomic DNA were 0.0125 and 0.05 µg μl^{-1} respectively. Clearly, these concentrations are similar to that one might expect in a human or cattle blood sample. The possibility that the technician might contaminate the reaction has also been discussed above, and one must not forget the potential problem of *S. mansoni* co-infection and cross reactivity with LAMP RIME.

While detection of all members of the subgenus *Trypanozoon* by LAMP seems inadequate other species specific LAMP assays also show promise. It is these to which we turn in the subsequent chapters, returning to a more general discussion of the suitability of LAMP technology per se in the final chapter.

4 Chapter 4. LAMP assays for the specific detection of *Trypanosoma brucei rhodesiense*

4.1 Introduction

The demand for new and improved diagnostics for *T. b. rhodesiense* is well illustrated by the Ugandan situation. Uganda alone reported more than half of the new cases of Rhodesian sleeping sickness in 2006; a total of 245 out of 486 cases (note that under-detection and under-reporting are major issues and any reported figures are deceptively low). Also in Uganda, if the northwards trade of infected animals continues unchecked the human *T. b. gambiense* and *T. b. rhodesiense* foci of sleeping sickness are predicted to merge, which would further complicate both diagnosis and treatment and compromise prognosis. Improved diagnostic tools for *T. b. rhodesiense* are therefore urgently required to improve case detection and treatment, to enable more accurate determination of disease burden for public health practitioners and policy makers at all levels and to enable targeted application of therapeutic and prophylactic agents in domestic livestock and to make risk assessments [61]. Rational control of Rhodesian sleeping sickness requires diagnostics that can be used at local centres to distinguish *T. b. gambiense* and *T. b. rhodesiense* [32].

The first, and only, LAMP assay for the specific detection of *T. b. rhodesiense* was published in 2008 [122], with primers targeting the *SRA* gene (referred to hereon as LAMP *SRA*1).

Four sequenced *SRA* genes are publically available for access through the GenBank database. Z37159 was the first sequenced, in 1994, from a Ugandan isolate [172]. Five years later AF097331 was independently identified in a Kenyan strain [169]. In 2002, the *SRA* gene from an additional eight *T. b. rhodesiense* isolates were sequenced, including two of Tanzanian origin, two Kenyan, two Zambian, one

Ugandan and one Ethiopian. Two new sequences were identified AJ345057 (the so called 'northern variant') identical to the Tanzanian, Kenyan and Ugandan *SRA* genes, and AJ345058 (the 'southern variant') found to be identical to the Zambian and Ethiopian *SRA* genes [52]. When compared, these genes were 97.9-99.7 % homologous with only three minor sequence variants. Sequences AJ345057 and AJ345058 are similar to AF097331 rather than Z37159, which is now thought to represent a unique mutation in the clone or parental line from which it was sequenced [52].

The LAMP *SRA*1 primers were designed using *SRA* sequence Z37159 and the assay was validated using DNA from 49 *T. b. rhodesiense* isolates (of which 14 were negative by a *SRA* PCR assay [52] and including isolates with the northern *SRA* gene and isolates with the southern variant *SRA* gene, as shown elsewhere [52]), five *T. b. gambiense*, six *T. b. brucei*, six *T. evansi*, three *T. congolense*, one *T. vivax*, one *T. simiae*, one *T. simiae* tsavo and one *T. godfreyi*. Njiru *et al.* [122] reported perfect diagnostic sensitivity and specificity, with a 1 pg detection limit.

Here, the LAMP *SRA*1 assay has been re-evaluated. The binding sites of the primers were mapped to all four databased *SRA* gene sequences. It was noted that these primers did not account for the deletion known to distinguish *SRA* from a multitude of similar VSG sequences. In light of this, and the *SRA* PCR negative-LAMP *SRA*1 positive results reported by Njiru *et al.* [122], the LAMP *SRA*1 assay was performed alongside the multiplex *SRA* PCR [108] reaction on 86 trypanosome DNA samples. The multiplex PCR is considered to be an improvement over previous PCR assays [32, 52] for *SRA* since it incorporates an internal amplification control that target the single copy trypanosomal *GPI-PLC* gene present in all *T. brucei* s.l. Novel LAMP primers were also designed for the *SRA* gene that did span the deletion site. The binding sites of these primers were mapped to all four *SRA* gene sequences available on the Genbank database. This chapter describes aspects of the molecular identification of DNA samples in this sample set, the re-evaluation of the published

LAMP assay for *T. b. rhodesiense*, and the design, optimisation and validation of a novel LAMP assay for *T. b. rhodesiense*.

4.2 Aims

1. To characterise the set of DNA samples using accepted PCR protocols as reference methods.
2. To validate the published LAMP primers and assay for *T. b. rhodesiense* by comparison to the reference multiplex PCR for *SRA*.
3. To design novel LAMP primers targeted to *SRA* accounting for its similarity to VSG genes.
4. To optimize and validate the novel LAMP assay on the DNA samples.
5. To review and compare sequenced *SRA* genes on database, mapping all LAMP primers to these genes in order to ensure the validity of these methods for all (northern and southern) *T. b. rhodesiense*s.

4.3 Study outline

Firstly, the 86 DNA samples extracted from cryo-preserved procyclic and bloodstream form trypanosomes were subject to:

- TCS PCR [188] as the standard molecular tool for the identification of *T. congolense* (savannah).
- TBR PCR [107] as the standard molecular tool for the detection and identification of *Trypanozoon* DNA.
- LAMP *PfrA* [140] as a positive control reaction for LAMP amplification of a single copy gene.
- The multiplex *SRA* PCR reaction was used as the standard molecular diagnostic method for detection of *T. b. rhodesiense* [108].

- The published LAMP for *T. b. rhodesiense* [122] (referred to as LAMP *SRA*1) with endpoint detection by gel electrophoresis and UV illumination.

A novel LAMP assay was designed to be specific for *T. b. rhodesiense*, by designing novel primers specific for the *SRA* gene and accounting for the deletion site compared with *VSG*. Primers were trialed using But135 and DO DNA using a range of $MgSO_4$ concentrations. After selection of the most appropriate primers the reaction mix was adapted for use in the real time turbidimeter by varying the concentration of dNTPs and $MgSO_4$. The assay was applied to the 86 samples in the real time turbidimeter before optimisation of the loop primer concentrations and reaction temperature were attempted.

Two methods for endpoint detection were tried; Loopamp fluorescence detection reagent and calcein with $MnCl_2$. Both were performed using 1/10, 1/100 and 1/1000 dilutions of sample 86, using the optimised mastermix with 0.8 μM loop primers and Triton X-100 containing LAMP buffer in a heating block. The product was removed for gel electrophoresis and UV illumination to confirm the result.

Cross tabulations were used to calculate sensitivity, specificity, negative and positive predictive values and Cohen's kappa statistic, as well as 95 % confidence intervals, the prevalence and bias indices, and the maximum attributable kappa to aid the interpretation of kappa. These calculations were performed to compare the reference *SRA* PCR to:

(i) the published LAMP *SRA1*assay
(ii) the novel SRALAMP_a assay (assessed by real time turbidimeter)

(iii) the novel SRALAMP_a assay (assessed by heating block and gel electrophoresis, in triplicate, with the comparison made for each repeat of the LAMP assay);

(iv) the novel SRALAMP_a assay with 0.8 and 2.4 μM loop primers on 1 in 10 dilutions of the DNA samples (assessed by real time turbidimeter)

Finally, the four databased *SRA* sequences (Z37159, AF097331, AJ345057 and AJ345058) were aligned using the ClustalW2 multiple sequence alignment program without making any changes to the default settings (www.ebi.ac.uk/Tools/clustalw2/index.html) [245] and the binding sites of the published and novel LAMP primers were mapped to the four aligned *SRA* sequences from the database by eye.

4.4 Results

4.4.1 Molecular assays

The results of the molecular assays, excluding the novel SRALAMP_a assay, for all 86 samples are shown in Appendix 8. The results of each assay and comparisons between them are shown in more detail in the following sections.

4.4.1.1 Trypanozoon specific PCR reaction

All samples, except for four, were positive by the TBR PCR. Samples 18, 21, 71, and 85 were TBR PCR negative. Samples 49, 71 and 85 were *T. congolense* savannah (confirmed by the TCS PCR [188]) and were therefore expected to be TBR negative. There were three unexpected results: sample 49 is false positive and samples 18 and 21 are false negative by TBR PCR. Since samples 18 and 21 are positive by the *TgsGP* PCR they are henceforth considered as *T. b. gambiense* despite their TBR negative status.

4.4.1.2 Trypanozoon specific LAMP reaction

Eighty one samples were positive by LAMP *PfrA*. The average time to the turbidity threshold for LAMP *PfrA* positives was 38 min 59 s. Negative samples were 56, 62, 71, 72 and 85. Again, sample 49 was positive. It may be a mixed infection, or it may have been cross-contaminated with *Trypanozoon* DNA during DNA extraction, handling or storage. *T. congolense* samples 71 and 85 were expected to be *Trypanozoon* negative. Samples 56, 62 and 72 were excluded from later analysis of the LAMP *SRA* assays.

4.4.1.3 T. b. rhodesiense specific PCR reaction

Multiplex *SRA* PCR gives rise to three bands. A band corresponding to *VSG* amplicon is observed at >1kb, a 669 bp band corresponds to specific amplification of the *SRA* gene and a 324 bp band corresponds to amplification of the *GPI-PLC* gene, as an internal control to confirm the presence of sufficient quality trypanosome DNA in the sample for PCR amplification of a single copy gene. *PLC* bands were observed for all but two of the samples (56 and 85). This seems to confirm that sample 56 lacks sufficient DNA for amplification, as per the LAMP *PfrA* reaction. Sample 85 was expected to be *Trypanozoon* negative which explains the lack of a *PLC* band. Samples 49 and 71 were also expected to be *Trypanozoon* negative, however weak *PLC* bands were seen for these samples suggesting that these DNA samples contain some *T. brucei* s.l. DNA as well as *T. congolense* savannah. VSG bands were seen for all but six of the samples (21, 22, 23, 56, 58, 85). Samples 21, 22, 23 and 58 were all confirmed *T. b. gambiense* Group 1. Thirty six of the 86 samples were *SRA* positive, of which two were unexpectedly positive for both the *SRA* and the *TgsGP* gene, one was unexpectedly *SRA* positive but not *TgsGP* PCR positive and one was unexpectedly *SRA* negative. These four anomalous results are summarised in Table 4.1. Cohen's kappa statistic demonstrates very good agreement ($\kappa = 0.926$; 95 % confidence interval 0.844 – 1.008; prevalence index 0.179; bias index 0.012)

between the *SRA* PCR and the 84 samples with known identity (see Appendix 2 for the identity and corresponding reference for all samples). Table 4.2 summarises the data from which this kappa statistic is calculated. Neither the prevalence nor bias indices suggest that this kappa value is artificially inflated or deflated.

Table 4.1. Anomalous *SRA* PCR and *TgsGP* PCR results

Sample ID	Reference	Species	Collection data	*SRA* PCR	*TgsGP* PCR
5. Dal069	[246]	*T. b. gambiense*	Human; Côte d'Ivoire;1981	+	+
8. Papol 264	[247]	*T. b. rhodesiense*	Cow; Uganda;1990	-	-
12. Biyamina	[248]	*T. b. gambiense*	Human; Sudan;1982	+	-
33. UGH	[247]	*T. b. rhodesiense*	Human; Uganda ; 1988	+	+

Table 4.2. The multiplex *SRA* PCR compared to previous identification as *T. b. rhodesiense* (see Appendix 2 for the identity and corresponding reference for all samples)

		***SRA* PCR**		
		+	-	**Total**
T. b. rhodesiense	+	33	1	34
	-	2	48	50
	Total	35	49	84

4.4.2 Validation of the existing assay

The LAMP *SRA1* assay [122] showed very good agreement (κ = 0.901; 95 % CI 0.807 – 0.996). The two by two table and summary statistics for the accuracy of LAMP *SRA*1 compared to the *SRA* PCR reference test are shown in Tables 4.3 and 4.4. Neither the prevalence (0.157) nor the bias (0.024) indices suggest that this kappa value is artificially inflated or deflated. The maximum attributable kappa is 0.951.

Table 4.3. Two by two table summarising the agreement between LAMP *SRA1* and *SRA* PCR

		LAMP *SRA1*		
		+	-	**Total**
***SRA* PCR**	+	33	3	36
	-	1	46	47
	Total	34	49	83

Table 4.4. Summary statistics for the agreement between LAMP *SRA*1 and *SRA* PCR

Kappa (95 % CI)	0.901 (0.807 - 0.996)
Sensitivity (95 % CI)	91.67 % (77.43 – 97.87 %)
Specificity (95 % CI)	97.87 % (87.86 – 99.99 %)
NPV (95 % CI)	93.88 % (82.85 – 98.52 %)
PPV (95 % CI)	97.06 % (83.78 – 99.99 %)

4.4.3 Development of the novel assay

For the development of a novel assay several candidate primer sets were evaluated, of which one was taken forward for LAMP assay development. This included adaptation for use with a real time turbidimeter, optimisation, and evaluation on the complete sample set.

4.4.3.1 Initial trial of novel primers

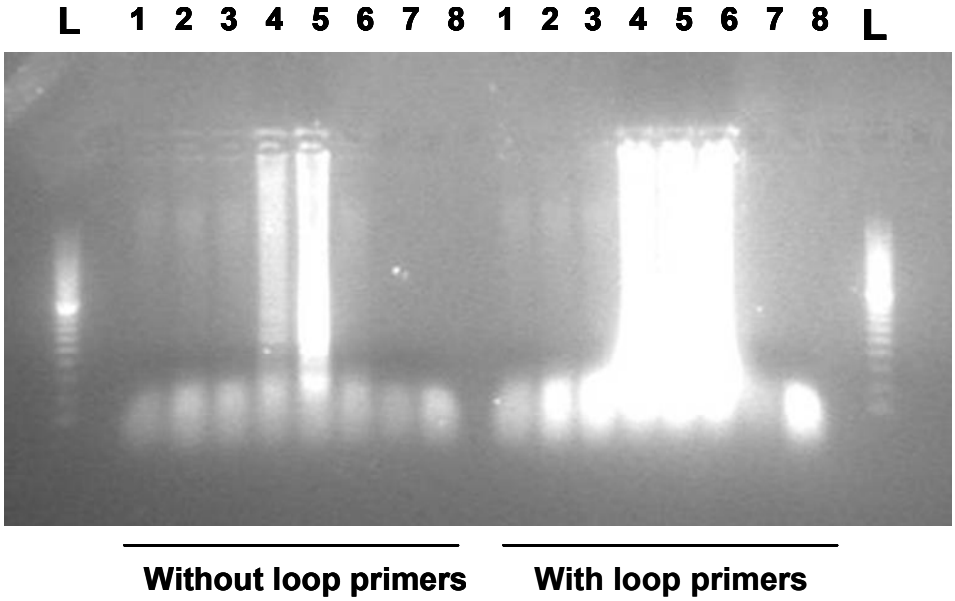
The SRALAMP_b, SRALAMP7.2 and SRALAMP7.10 primers amplified neither *T. b. rhodesiense* nor *T. b. brucei* at any of the concentrations of $MgSO_4$ at which they were tested.

At 2 mM $MgSO_4$, the SRALAMP_a primers specifically amplified *T. b. rhodesiense* but not *T. b. brucei*. Amplification occurred both with and without the loop primers,

using 1 µl of purified DNA as template. Without loop primers two out of three reactions seeded with DO (*T. b. rhodesiense*) were positive, however, when the loop primers were included all three were positive.

LAMP products were much brighter under UV light, as assessed by eye, when the loop primers were included. Thus it seemed that the loop primers enhanced the amplification efficiency of the reaction. Figure 4.1 shows the electrophoresed LAMP product, as seen under UV light, generated by the SRALAMP_a primers with and without loop primers, in the initial screen.

Figure 4.1. SRALAMP_a primers at 2 mM $MgSO_4$.



Lanes 1-3 But135 (*T. b. brucei*); lanes 4-6 DO (*T. b. rhodesiense*); lanes 7, 8 negative controls; lanes labelled L contain a 1 kb ladder.

The SRALAMP_e primers specifically amplified *T. b. rhodesiense* but not *T. b. brucei* at 2 mM, 4 mM, 6 mM and 8 mM $MgSO_4$. The LAMP product, as seen under UV light, were brightest at 4 mM, 6 mM and 8 mM $MgSO_4$, and were approximately equally bright at these concentrations.

The SRALAMP_f primers specifically amplified *T. b. rhodesiense* but not *T. b. brucei* at 2 mM, 4 mM, 6 mM and 8 mM $MgSO_4$. The ladders of bands were approximately equally bright at all these concentrations.

In the initial screen, at 2 mM $MgSO_4$, the SRALAMP7.1 primers neither amplified *T. b. brucei* nor *T. b. rhodesiense*. However, when the $MgSO_4$ concentration was altered specific amplification of *T. b. rhodesiense* was observed. Positive results were

observed for all *T. b. rhodesiense* samples, but no *T. b. brucei samples*, at 3 mM, 3.5 mM, 4 mM, 4.5 mM and 5 mM $MgSO_4$. The LAMP product appeared brightest, under UV light, for 3 mM, 3.5 mM or 4 mM $MgSO_4$ and less bright at 4.5 mM and 5 mM $MgSO_4$.

The SRALAMP7.3 primer set specifically amplified *T. b. rhodesiense* in the initial screen at 2 mM, 4 mM, 6mM and 8mM, with optimal amplification observed at 4 mM.

The SRALAMP7.13 primer set specifically amplified *T. b. rhodesiense* in the initial screen at 2 mM, 4 mM, 6 mM and 8 mM, with optimal amplification observed at 4 mM.

SRALAMP_a was taken forward as a potentially *T. b. rhodesiense* specific LAMP primer set for full validation. Full validation was not performed simultaneously for all primer sets, even though several others showed promise, simply because the samples set was finite and time and resources were limited.

4.4.3.2 Adaptation of SRALAMP_a for use with the real time turbidimeter

When the concentration of $MgSO_4$ and dNTPs were simultaneously increased to 8 mM and 1.4 mM respectively, positive LAMP reactions were seen specifically for four *SRA* positive samples (32, 36, 64, 65 and 68) but not for two *SRA* negative samples (24 and 28) (both on a gel and using the turbidimeter).

4.4.3.3 Optimisation of the reaction conditions for the novel assay

To see if the time to reach the turbidity threshold could be reduced, and to make the reaction as sensitive as possible, the loop primer concentration and incubation temperature were tested over a range of reasonable values.

When loop primers were included into the reaction at a 0.8 μM, which is a typical concentration for loop primers in a LAMP reaction, intermediate between the concentrations of the inner and outer primers, the LAMP reaction was more sensitive and more efficient than when no loop primers were used. The detection limit improved by a factor of five, from a 1/1000 dilution (0.0117 ng DNA) to a 1/5000 dilution (0.0234 ng DNA) and the reaction was 23 min 36 s faster on average compared to when no loop primers were used. The detection limit and reaction efficiency were improved again when the loop primer concentration was increased to 2.4 μM, which is in excess of the inner primer concentration. With 2.4 μM loop primers the detection limit improved from a 1/5000 dilution (0.0234 ng DNA) to a 1/10000 dilution (0.00117 ng DNA) and the reaction was 7 min 47 s faster on average compared to when 0.8 μM loop primers are used. These data are shown in Table 4.5.

Table 4.5. Effect of loop primers on the sensitivity and efficiency of the SRALAMP_a reaction

Dilution factor	Time taken to reach the turbidity threshold		
	Without loop primers	Loop primers at 0.8 μM	Loop primers at 2.4 μM
1/10	01:00:54	00:37:48	00:29:30
1/50	01:02:30	00:38:30	00:31:30
1/100	01:02:42	00:39:12	00:31:24
1/500	01:09:36	00:42:18	00:32:54
1/1000	01:15:06	00:55:00	00:39:42
1/5000	-	00:58:00	00:59:06
1/10000	-	-	00:64:18

Given the reductions seen in reaction time and detection limit when loop primers were used at 2.4 μM the assay was re-evaluated. Keeping everything else the same the assay was applied to 1/10 dilutions of the sample DNAs, firstly using 0.8 μM and secondly using 2.4 μM loop primers. Samples 21 and 72 were excluded from the analysis because they were LAMP *PfrA* negative at this dilution. When 2.4 μM loop primers were used the agreement with the reference PCR was reduced from κ = 0.852 (very good) to κ = 0.354 (fair) and the specificity of the assay was largely lost, falling from 92.50 % to 42.50 %. Neither the prevalence nor bias indices suggest that this kappa value is artificially inflated or deflated, although the bias index is higher for the 2.4 μM screen. There is no overlap in the 95 % confidence intervals, which supports the conclusion that 2.4 μM loop primers worsens the agreement between the LAMP assay and the reference PCR. These calculations were made from the data shown in Table 4.6 below. The diagnostic accuracy summary statistics at both loop primer concentrations are shown in Table 4.7 below.

Table 4.6. Two by two table comparing SRALAMP_a with the reference PCR at two different concentrations of loop primers

		SRALAMP_a					
		0.8 µM loop primers			**2.4 µM loop primers**		
		+	-	**Total**	+	-	**Total**
***SRA* PCR**	+	27	2	29	28	1	29
	-	3	37	40	23	17	40
	Total	30	39	69*	51	18	69*

*samples 2 and 73-86 were not done

Table 4.7. Summary statistics for SRALAMP_a using different concentrations of loop primers compared to the reference *SRA* PCR

	0.8 µM loop primers	**2.4 µM loop primers**
Kappa	0.852	0.354
(95 % CI)	(0.727, 0.977)	(0.154, 0.562)
Sensitivity	93.10 %	96.55 %
(95 % CI)	(76.97 – 99.15 %)	(81.37 – 99.99 %)
Specificity	92.50 %	42.50 %
(95 % CI)	(79.43 – 98.12 %)	(28.50 – 57.82 %)
NPV	94.87 %	94.44 %
(95 % CI)	(82.21 – 99.48 %)	(72.35 – 99.99 %)
PPV	90.00 %	54.90 %
(95 % CI)	(73.58 – 97.34 %)	(41.38 – 67.74 %)

To aid interpretation of kappa the prevalence and bias indices and the maximum attributable kappa were also calculated (Table 4.8).

Table 4.8. Prevalence and bias indices, as well as the maximum attributable kappa for the data in Tables 4.6 and 4.7.

	0.8 µM loop primers	**2.4 µM loop primers**
κ_{max}	0.970	0.407
Prevalence index	0.145	0.159
Bias index	0.014	0.319

The reaction time was fastest at 60 ˚C or 62 ˚C depending on the concentration of DNA used as template for the LAMP reaction. Generally when higher concentrations of DNA were used the reaction was most efficient at 62 ˚C; at lower concentrations of DNA the reaction was most efficient at 60 ˚C. The reaction times at each temperature over the dilution series are shown in Table 4.9 below (the fastest reaction time at each DNA concentration is highlighted in red). Over the temperature range 58 ˚C to 62 ˚C the detection limit of the assay was not reached. At 64 ˚C and 65 ˚C the detection limit is not clear cut, but these results suggest that the assay may be less sensitive at these higher temperatures.

Table 4.9. Effect of temperature on the efficiency and sensitivity of the SRALAMP_a assay

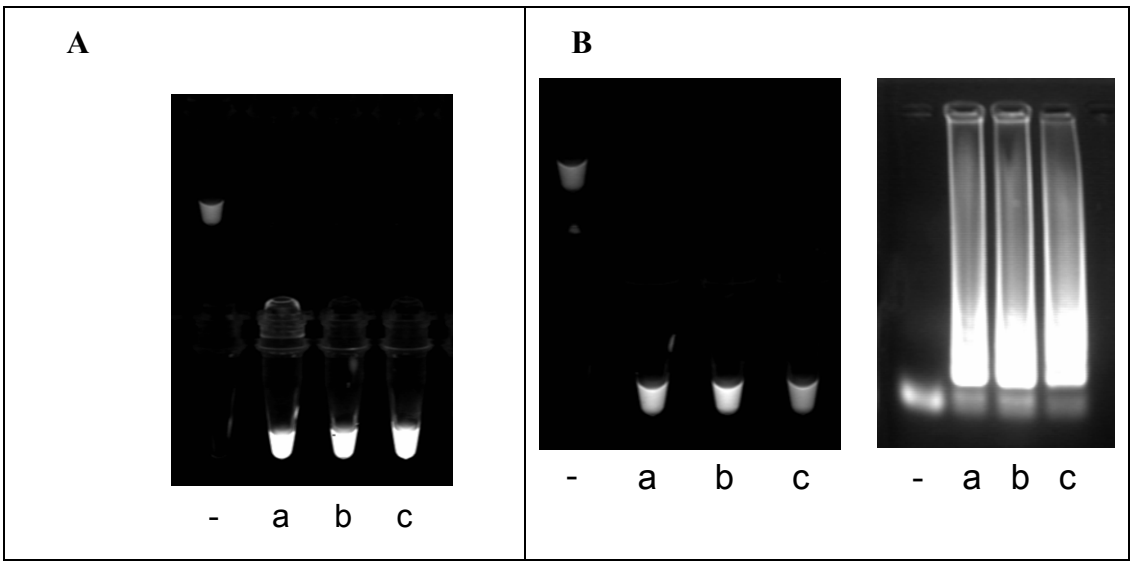
Dilution factor	Time taken to reach the turbidity threshold				
	58 ˚ C	60 ˚C	62˚ C	64˚ C	65˚ C
1/10	00:51:30	00:43:12	00:42:00	00:48:12	01:05:06
1/50	00:54:24	00:45:30	00:45:18	00:52:48	01:08:24
1/100	00:54:36	00:47:36	00:46:48	00:55:00	01:03:42
1/500	00:59:00	00:51:24	00:53:00	01:04:00	01:09:30
1/1000	01:03:06	00:53:30	00:51:54	-	01:12:12
1/5000	01:05:36	00:54:36	00:58:36	01:14:00	-
1/10000	01:05:30	01:02:12	01:13:18	-	01:16:48

4.4.3.4 Endpoint detection

Figure 4.2 panel A shows the fluorescence, under UV illumination, when Loopamp Fluorescence Detection Reagent was used with SRALAMP_a. In panel A the fluorescence emitted by a negative control is compared to the fluorescence emitted from three positive reactions (a, b and c, which each contained a different concentration of template DNA). Regardless of the DNA concentration used to seed the SRALAMP_a reaction fluorescence appeared brighter than the negative control. Under ambient light a detectable colour change was also discernible by eye.

In contrast, Figure 4.2 panel B shows the fluorescence, under UV illumination, when calcein and $MnCl_2$ were used with SRALAMP_a. The gel electrophoresis results are adjacent. Clearly, all three reactions (a, b and c) are positive, and yet the fluorescence is difficult to distinguish from the negative control, particularly for tube c which was seeded with the lowest concentration of DNA.

Figure 4.2. Assessing the endpoint of the SRALAMP_a assay at 0.8 µM loop primers with Thermopol reaction buffer



Panel A. Loopamp Flourescence Detection Reagent with a) 1/10 dilution of DO, b) 1/100 dilution, c) 1/1000 dilution and panel B. Calcein and $MnCl_2$, as per Tomita , Mori *et al.* [136] with a) 1/10 dilution of DO, b) 1/100 dilution, c) 1/1000 dilution.

4.4.4 Evaluation of the novel assays

When it was performed and assessed using the real time turbidimeter the SRALAMP_a assay showed very good agreement (κ = 0.808; 95 % CI 0.681 - 0.934) with the *SRA* PCR assay. The average time to the turbidity threshold was 39 min 48 s. For true positives only the average time was 38 min 0 s. The average time for the false positives was 48 min 50 s. Next, the SRALAMP_a was performed in triplicate, in a thermocycler, followed by optical turbidity and gel electrophoretic assessment. Tables 4.10 and 4.11 present the two by two comparison for SRALAMP_a versus *SRA* PCR using the real time turbidimeter and the heating block respectively.

Table 4.10. SRALAMP_a versus *SRA* PCR (real time turbidimeter)

		SRALAMP_a		
		+	-	**Total**
***SRA* PCR**	+	35	1	36
	-	7	40	47
	Total	42	41	83

Table 4.11. SRALAMP_a, incubated in a thermocycler and assessed by gel electrophoresis, applied three times to the sample set, versus *SRA* PCR

		Replicate of SRALAMP_a								
		1			**2**			**3**		
		+	-	**Total**	+	-	**Total**	+	-	**Total**
***SRA* PCR**	+	34	2	36	34	2	36	34	2	36
	-	2	45	47	5	42	47	11	36	47
	Total	36	47	83	39	44	83	45	38	83

Table 4.12 presents the summary statistics comparing SRALAMP_a to the PCR for each test. When the SRALAMP_a, with the results read by gel electrophoresis, was compared to the *SRA* PCR results for these samples, the agreement, measured by Cohen's kappa statistic, ranged from $\kappa = 0.690$ to $\kappa = 0.902$. There is considerable overlap between the 95 % confidence intervals for these three kappa values, supporting the hypothesis that this is a reliable assay.

Table 4.12. Summary statistics for the comparison of the index test, SRALAMP_a, and the reference test *SRA* PCR

	Real time turbidimetry	Gel electrophoresis		
		Repeat of SRALAMP_a		
		1	2	3
Kappa **(95% CI)**	0.808 (0.681, 0.934)	0.902 (0.808, 0.996)	0.830 (0.715, 0.952)	0.690 (0.536, 0.845)
Sensitivity **(95% CI)**	97.22 % (84.58 – 99.99 %)	94.44 % (80.91 – 99.41 %)	94.44 % (80.91 – 99.41 %)	94.44 % (80.91 – 99.41 %)
Specificity **(95% CI)**	85.11 % (72.00 – 92.91 %)	95.74 % (84.96 – 99.62 %)	89.36 % (76.96 – 95.81 %)	76.60 % (62.62 – 86.55 %)
NPV **(95% CI)**	97.56 % (86.26 – 99.99 %)	95.74 % (84.96 – 99.62 %)	95.45 % (84.03 – 99.58 %)	94.74 % (81.80 – 99.46 %)
PPV **(95% CI)**	83.33 % (69.08 – 92.00 %)	94.44 % (80.91 – 99.41 %)	87.18 % (72.83 – 94.87 %)	75.56 % (61.18 – 85.92 %)

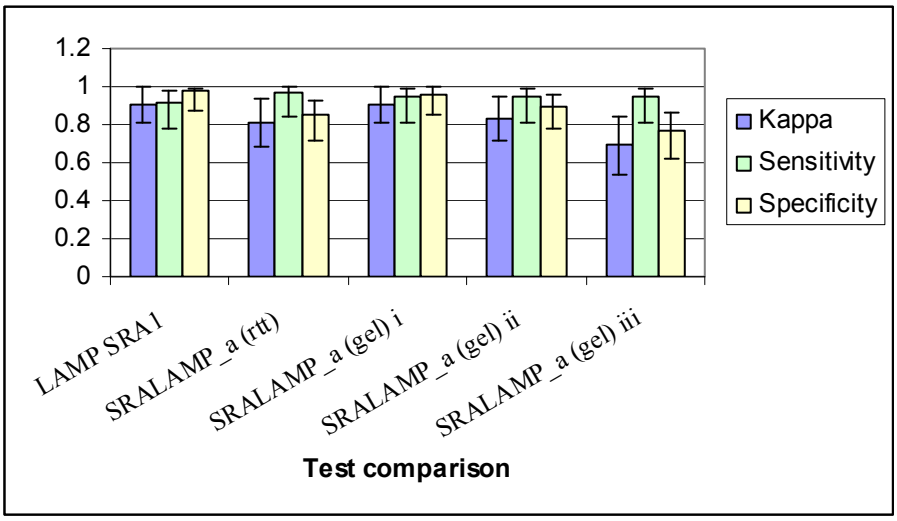
To aid interpretation of kappa the prevalence and bias indices and the maximum attributable kappa were also calculated (Table 4.13).

Table 4.13. Prevalence and bias indices, as well as the maximum attributable kappa for the data in Tables 4.11 and 4.12.

	Real time turbidimetry	Gel electrophoresis		
		1	**2**	**3**
κ_{max}	0.856	1	0.927	0.786
Prevalence index	0.060	0.133	0.096	0.024
Bias index	0.072	0	0.036	0.108

Overall the best performance of the SRALAMP_a assay was the first screen of the sample set in the thermocycler, as assessed by gel electrophoresis when very good agreement ($\kappa = 0.902$) was seen, with the LAMP test being 94.44 % sensitive and 95.57 % specific as compared to the PCR. All the results from the SRALAMP_a assay on the total sample set are given in Appendix 9. An assessment of the reliability of this assay, using these results is addressed in Chapter 7 and the usefulness of turbidity as a simple visual endpoint detection method is analysed and discussed in Chapter 8.

Figure 4.3. Kappa, sensitivity and specificity estimates, with 95 % CI for LAMP SRA1 and SRALAMP_a compared to the reference *SRA* PCR



SRALAMP_a (rtt) is performed using the real time turbidimeter; SRALAMP_a (gel) is incubated in a thermocycler with endpoint detection via gel electrophoresis and UV illumination, this was repeated three times (i, ii and iii)

4.4.5 Multiple sequence alignment of databased *SRA* sequences

Multiple sequence alignment of the four databased *SRA* sequences using the ClustalW2 program (www.ebi.ac.uk/Tools/clustalw2/index.html) [245] is shown in Figure 1, Appendix 10. There were 28 mismatches across the total sequence.

4.4.6 Comparison of LAMP primer binding sites with northern and southern variant *SRA* sequences

4.4.6.1 Published LAMP primers

Perfect consensus was seen between the published LAMP primers, which were designed using the original Ugandan *SRA* gene Z37159, to northern variant *SRA* gene

AJ345057. However two separate single nucleotide mismatches were observed when the same primers were compared to the southern variant *SRA* gene sequence AJ345058, of which one was located in the F3 primer, and one was located in the FIP primer (Figure 2, Appendix 10).

4.4.6.2 Novel LAMP primers

Perfect consensus was seen between the novel LAMP primers, designed for the *SRA* gene AF097331, to northern variant *SRA* gene AJ345057 and southern variant *SRA* gene AJ345058 (Figure 3, Appendix 10).

4.5 Discussion

In 1989 the gene which confers human infectivity in *T. b. rhodesiense* – the *SRA* gene – was discovered [24] (independently confirmed in 1998 [170]). The *SRA* gene has proved to be a ubiquitous and conserved genetic marker for *T. b. rhodesiense* [52]. Its discovery and characterisation has enabled sensitive and specific molecular detection of this parasite, which in turn has greatly enhanced our understanding of the epidemiology of Rhodesian sleeping sickness. In particular cattle have been identified as important reservoir hosts, with a role in spreading disease into new geographic areas [32, 33].

Several PCR assays have been developed for *T. b. rhodesiense*, each with primers that target the *SRA* gene [33, 52, 108, 175]. When designing PCR primers for *SRA* it is critical that they account for the deletion characteristic [108]: the *SRA* gene has apparently arisen as a result of a 378 bp deletion within what was originally a VSG gene and the putative breakpoints for this deletion are conserved [172, 173]. If this is not taken into consideration primers will likely bind VSG genes also present in other *T. brucei* s.l. species. This is made apparent by the amplified VSG band in the multiplex *SRA* PCR [108].

For this study the *multiplex* PCR [108] was chosen for use as the reference standard for the molecular detection of *T. b. rhodesiense*. This multiplex PCR reaction includes contains an internal control which indicates whether sufficient genomic material is present for detection of a single copy *T. brucei* gene. The multiplex *SRA* PCR showed very good agreement ($\kappa = 0.926$) with previous identification as *T. b. rhodesiense* (these previous identifications were made according to a variety of methods, as described and referenced in Appendix 2). Two false positives (both supposed to be *T. b. gambiense*) and one false negative were observed. One of the false positives (sample 12) was previously *SRA* PCR positive using a different DNA extraction, so this is probably a true identification. The false negative might also be a correct identification. This isolate was identified as *T. b. rhodesiense* according to RFLP analysis, however, human serum sensitivity in vitro has not been tested for this isolate, which was collected from a cow. Despite discrepancies it was reasonable to use the PCR as a molecular test against which to validate the published and new LAMP assays for *T. b. rhodesiense*.

In 2008 a LAMP assay for the specific detection of *T. b. rhodesiense* was developed, whose primers were targeted to the *SRA* gene [122]. However, these primers bind upstream of the conserved *SRA* specific deletion site. Therefore, it was feared that the assay might also amplify *T. b. brucei* and *T. b. gambiense* DNA. Amplification of an unintended target is potentially more serious with LAMP than with traditional PCR since the majority of LAMP endpoint detection methods are not sequence specific. PCR products are usually confirmed by the size of the amplified fragment, which is seen upon UV illumination of the reaction products after they have been separated by gel electrophoresis. However LAMP readout is typically positive or negative. When LAMP products are separated by gel electrophoresis, a ladder of bands is seen, but it is the presence rather than the shape of the ladder that is usually used for detection.

Sequence mismatches were also observed within the F3 and FIP primers when compared to the southern variant *SRA* sequence. Together these observations justified re- validation of the assay using a different set of DNA samples.

The published assay for *T. b. rhodesiense* showed very good agreement ($\kappa = 0.901$) with the multiplex *SRA* PCR, detecting 92 % of *SRA* positive samples and showing 98 % specificity. It was not possible to fully validate its application to the 'southern' *T. b. rhodesiense* as samples were not available.

Although the published assay unexpectedly showed very good agreement with the multiplex *SRA* PCR reaction, novel primers were also designed which accounted for the *SRA* specific internal deletion site.

Initially all novel primers were tested on one *T. b. rhodesiense* and one *T. b. brucei* DNA sample. The same reaction conditions were selected that had been used for the LAMP *SRA*1 assay (62 ˚C for 1 h followed by 4 min at 80 ˚C) and the composition of the reaction mix was the same as for the published LAMP RIME [121] and *SRA*1 [122] reactions (Chapter 2). However, after the SRALAMP_a assay was selected for further validation this was modified with supplementary $MgSO_4$, and additional dNTPs, so that these reagents were present in the same concentrations as in the published LAMP *PfrA* and TBG1 reactions [140, 141] in order to use turbidity for endpoint detection, both visually and using a real time turbidimeter. The sample set was screened four times using the SRALAMP_a assay. Firstly the assay was performed in the real time turbidimeter, which incubates the reaction, and gives a readout signal which indicates the time at which turbidity surpasses a predefined threshold. Then the assay was performed three times in a heating block after which the endpoint was detected by a visual assessment of the turbidity, and by gel electrophoresis with UV illumination.

Using the real time turbidimeter, the SRALAMP_a assay showed very good agreement (κ = 0.808) with the multiplex *SRA* PCR assay. It detected 97 % of *SRA* PCR positives and was 85 % specific. Noticeably, the agreement between the novel LAMP assay and the PCR is less good than for the published assay and the PCR. The novel assay is more sensitive, but less specific. In other words it is detecting more true positives, but also more false positives.

The average time to reach the turbidity threshold was approximately the same as for the LAMP *PfrA* assay on the same samples, but is not so fast as some reported LAMP assays (e.g. less than 30 minutes [125]). Attempts to improve the reaction efficiency were made during this study; variations in the concentration of loop primers and the incubation temperature were both trialled. At first it seemed that by increasing the concentration of loop primers the efficiency might be dramatically improved, however this came with an unacceptable decrease in reaction specificity. The temperature remained optimal at 62 ˚C. It might still be possible to further improve the efficiency of the reaction: small alterations in the primer binding site have been shown to have dramatic effects on reaction efficiency during the development of other LAMP assays (Chie Nakajima, personal communication). Finally, small adjustments in the composition of the reaction mix, or the incubation temperature might also improve the efficiency of the reaction.

Since the LAMP *SRA*1 assay was not assessed in the turbidimeter it is not possible to make direct comparisons of reaction efficiency for these two assays.

The first time the SRALAMP_a assay was applied to the full sample set using a heating block the results showed very good agreement with the multiplex *SRA* PCR (κ = 0.902). This agreement was comparable to that seen with the published LAMP reaction, and was an improvement over the same assay performed and assessed by real time turbidimeter. Furthermore, the sensitivity and specificity were both high (94

% and 95 % respectively). Fewer false positives are detected when DNA amplicon is visualised directly via gel electrophoresis, than by turbidity, which is a by-product of DNA amplification.

Repeatability of both the published and novel assays are analysed and discussed in Chapter 7. However, here at first glance alone we can see that the agreement of the novel SRALAMP_a assay over several repeats is far from perfect. This is possibly due to degradation of the DNA samples and the increasing likelihood of cross contamination between samples over time and with repeated use.

Njiru *et al.* report that their LAMP assay (LAMP *SRA*1) is 100 times more sensitive than PCR for the *SRA* gene [122]. Using 10 fold serial dilutions of 100 ng DNA, pre heated at 96 ˚C for 1 min, they could reliably detect 1 pg DNA with the LAMP *SRA*1 but only 100 pg DNA by PCR [52] (not the multiplex *SRA* PCR used in this study). The multiplex PCR can reliably differentiate between the *T. brucei* subspecies using genomic DNA diluted to the equivalent of one trypanosome (0.12 pg) [108]. The novel SRALAMP_a assay here could detect up to 1.17 pg diluted genomic DNA (approximately equivalent to 10 trypanosomes). Therefore, the novel LAMP assay is roughly equivalent to the published assay. Following the approach of Njiru *et al.* [122] sensitivity of SRALAMP_a might be improved by pre-heating the template.

Simple, closed tube, endpoint detection would constitute an enormous benefit for a diagnostic intended for use in a resource poor, low technology setting. Turbidity was not reported for the published LAMP *SRA*1 assay, nor were any other closed tube detection methods applied to the LAMP *SRA*1 assay in the original study. In this study, turbidity ‘by-eye’ was possible for SRALAMP_a. Its accuracy compared to the results by gel electrophoresis for the same LAMP amplifications is described, analysed and discussed in Chapter 8. In summary, 79 % of positive SRALAMP_a reactions could be detected by turbidity, without any false positives. However,

turbidity assessment is not objective, but depends upon the reader's interpretation of what they see, and here, turbidity assessments were made by one relatively experienced observer. An observer who selected a less stringent threshold for scoring a reaction as turbid would be likely to detect more false positives. Also, the agreement between turbidity, 'by-eye' and gel electrophoresis, was not consistent. Therefore, although 'by-eye' turbidity might be useful as a quick and dirty, technology-free method for reading LAMP results it is too subjective and insensitive to be used for definitive diagnosis. It is possible that simple yet quantitative endpoint turbidity measurement might be useful in field settings. Further work measuring the turbidity of LAMP reactions, followed by receiver operating curve analysis, would be useful to determine whether quantitative endpoint turbidimetry could be reliably used to discern LAMP positive and negative reactions.

The small amount of preliminary work performed here suggested that the commercially available Loopamp flourescence detection reagent would enable discrimination of positive and negative endpoints for SRALAMP_a by a colour change that is visible by eye and according to fluorescence under UV. However this was only performed on a very few samples, and requires further validation. According to the manufacturers it should be stored at -20 ˚C, which may act as a barrier to use in HAT endemic areas owing to the cold chain requirement. Endpoint detection is discussed in detail in Chapter 8.

4.5.1 Conclusions

Both the published and novel LAMP assays designed to specifically detect *T. b. rhodesiense* enabled very good, but imperfect, agreement with the reference standard molecular diagnostic, the multiplex *SRA* PCR assay. Using data generated from the first full screen of the total sample set for each assay, performed in a thermocycler, followed by gel electrophoresis with UV visualisation, agreement with the reference standard PCR was almost identical for the two assays, the sensitivity was marginally

higher with the novel assay, whereas the specificity of the published assay was slightly better (Table 4.16).

Table 4.16. Comparing the diagnostic sensitivity and specificity of the two *T. b. rhodesiense* LAMP assays

	LAMP assay	
	SRALAMP_a (novel)	**LAMP *SRA*1 (published)**
Kappa	0.902	0.901
Sensitivity	94.44 %	91.67 %
Specificity	95.57 %	97.87 %

However this comparison hides a lack of LAMP assay reproducibility which is assessed in more detail in Chapter 7 and makes direct comparison of the two assays difficult. Each assay has its own advantages. The published assay probably has a lower detection limit, whereas the novel assay has been optimised to enable simple endpoint discrimination by eye, using turbidity. Since the published assay does not give rise to turbidity in the reaction tube of positive reactions it might be difficult to perform in a real time turbidimeter. Given that the novel assay can be performed in a real time turbidimeter it is in a better position for further optimisation. Both assays have potential for further development as field friendly molecular diagnostic tools for *T. b. rhodesiense*. The future of these assays depends on further validation studies, and improvements to sample preparation and endpoint visualisation.

5 Chapter 5. Evaluation of a LAMP protocol [141] designed for the specific detection of *Trypanosoma brucei gambiense*

5.1 Introduction

T. b. gambiense classically falls into two groups first differentiated by isoenzyme and restriction fragment length polymorphism analysis [248, 251-256] and later distinguished by unique microsatellites [257-259] and sequence polymorphisms within the 5.8S rRNA ITS region [249]. Most *T. b. gambiense* that have been isolated are from the genetically homogenous Group 1 type [248, 256, 260-262] with the remainder classified as Group 2.

The number of reported cases for *T. b. gambiense* fell to 9877 by 2009 [59], the currently available diagnostics for *T. b. gambiense* remain far from perfect (see Chapter 1).

At the start of this study the only LAMP assay for the specific detection of *T. b. gambiense* was published in 2007 [141]. This targeted the 5.8S rRNA - internal transcribed spacer 2 (ITS2) gene using primers that were designed for the sequence with accession number AF306777 (referred to as LAMP TBG1).

The AF306777 sequence spans the ITS-1, ITS-2 and intervening 5.8S rRNA of a *T. b. gambiense* group 2 isolate known as TH2 (78E) originating from a human infection in Koudougou, Côte d'Ivoire. This region is supposed to be largely non functional and to be under minimal selective pressure. It was sequenced as part of a study conducted by Agbo *et al.* in 2001 [249], to investigate whether polymorphisms in this region could be used to differentiate *T. b. brucei* and *T. b. gambiense*. Agbo *et al.* used restriction fragment length polymorphisms (RFLP) (on six *T. b. brucei* and

seven *T. b. gambiense* isolates, of which one was Group 2) and sequence analysis (on four *T. b. brucei* and four *T. b. gambiense* isolates, of which one was Group 2). The RFLP analysis supported segregation of the isolates into three groups; one containing five *T. b. brucei* isolates, a second containing six *T. b. gambiense* Group 1 isolates and a third containing the one *T. b. gambiense* Group 2 isolate. Sequence alignments identified a unique four base pair C_3A insertion in the 5.8S rRNA gene of all four *T. b. gambiense* isolates.

Thekisoe *et al.* [141], validated LAMP TBG1 using DNA extracted (by a standard method [250]) from *in vitro* parasite cultures of nine *T. b. gambiense* strains (five Group 1 and four Group 2 strains), one *T. b. brucei*, one *T. b. rhodesiense*, two *T. congolense*, one *T. cruzi* and nine *T. evansi*. Several non-trypanosomal parasite cultured samples were also used (*Babesia bovis*, *B. bigemina*, *B. caballi*, *B. equi*, *Theleria orientalis*, *T. parva*, *Toxoplasma gondii* and *Neospora caninum*). With this sample set, the LAMP TBG1 primers were shown to be highly specific for *T. b. gambiense* and could detect as little as 1 fg of *T. b. gambiense* DNA. Both the sensitivity and specificity assays were reported to be consistent when repeated in pentaplicate [141].

In the present work, LAMP TBG1 was applied to a larger set of *Trypanozoon* DNA samples in comparison to *TgsGP* PCR as a reference test for this subspecies.

5.2 Aims

There were initially two main aims to this study:

1. To screen the sample set with the reference PCR for the specific detection of Group 1 *T. b. gambiense*.
2. To validate the published LAMP primers and assay for *T. b. gambiense* on a larger set of *Trypanozoon* DNA samples.

As the results were analysed it became apparent that it was also necessary to:

3. Investigate the causes of observed non specificity in the published primers.

5.3 Study outline

Firstly, the 86 DNA samples extracted from cryo-preserved procyclic and bloodstream form trypanosomes were subject to:

- TCS PCR [188] as the standard molecular tool for the identification of *T. congolense* (savannah).
- TBR PCR [107] as the standard molecular tool for the detection and identification of *Trypanozoon* DNA.
- *TgsGP* PCR [32, 109] reaction as the standard molecular diagnostic method for detection of *T. b. gambiense*.
- LAMP *PfrA* [140] as a positive control reaction for LAMP amplification of a single copy gene.
- LAMP TBG1[141] performed twice per sample; once in a heating block and once in a real time turbidimeter

Cross tabulations were then used to calculate sensitivity, specificity, negative and positive predictive values and Cohen's kappa statistic, as well as 95 % confidence intervals, the prevalence and bias indices, and the maximum attributable kappa to aid the interpretation of kappa. These calculations were performed to compare:

(i) Published identity as a Group 1 *T. b. gambiense* against *TgsGP* PCR

(ii) Published identity as *T. b. gambiense* (Group 1 or Group 2) against
   a. LAMP TBG1 (assessed by gel electrophoresis)
   b. LAMP TBG1 (assessed by real time turbidimetry);

(iii) *TgsGP* PCR against
   a. LAMP TBG1 (assessed by gel electrophoresis)
   b. LAMP TBG1 (assessed by real time turbidimetry);

(iv) LAMP TBG1 (assessed by gel electrophoresis) against LAMP TBG1 (assessed by real time turbidimetry).

Thirdly, the LAMP TBG1 primers were compared to their target sequence in four *T. b. brucei* and four *T. b. gambiense* sequences for the target region which were described by Agbo *et al.* [249] (Accession numbers AF306770, AF306771, AF306772, AF306773, AF306774, AF306775, AF306776 and AF306777). The position of the primers in relation to the *T. b. gambiense* specific C_3A insertion was also determined.

5.4 Results

The results of the four molecular assays (TBR PCR, LAMP *PfrA*, *TgsGP* PCR and LAMP TBG1), for all 86 samples are fully tabulated in Appendix 12. The results from the TBR PCR and LAMP *PfrA* were fully described in Chapter 4. A key point to note here is that three samples (56, 62 and 72) were excluded from all analyses because they were LAMP *PfrA* negative, despite being TBR PCR positive. The results from *TgsGP* PCR and LAMP TBG1 as well as the comparisons between made between them are described in detail in the following sections.

5.4.1 *T. b. gambiense* specific PCR reaction

TgsGP PCR was positive for nine samples but these were not all the same nine classified as in Group 1 *T. b. gambiense* in the scientific literature [246, 248, 252] (see Appendix 11). A two by two table is shown below (Table 5.1) which excludes LAMP *PfrA* negative samples. *TgsGP* PCR shows good agreement [199] ($\kappa = 0.803$; 95 % CI 0.585-1.022) with previous identification as Group 1 *T. b. gambiense* and is

highly specific (97.3%; 95 % CI 90.23-99.83 %), though less sensitive (87.5%; 95 % CI 50.78-99.89 %). The confidence interval for sensitivity is wide, owing to the small number of *T. b. gambiense* samples. The prevalence index is high (0.795), again because of the small number of *T. b. gambiense* samples, which may lead to an artificial reduction in kappa. However, the bias index is low (0.012).

Table 5.1. *TgsGP* PCR agreement and earlier speciation of the isolates

	***TgsGP* PCR**		
Published identity	+	-	**Total**
Group 1 *T. b. gambiense*	7	1	8
Not Group 1 *T. b. gambiense*	2	73	75
Total	9	74	83

Information about the discrepant samples is given in Table 5.2. This includes one false negative (highlighted in green) and two false positive samples (blue), as well as one LAMP *PfrA* negative sample which may also be a false negative (pink), and a fifth sample which was correctly identified by the *TgsGP* PCR assay, but was also positive by the *SRA* PCR.

Table 5.2. Discrepancies between the *TgsGP* PCR and published sample identities.

Sample ID	Ref.	Species	Collection data	*SRA* PCR	*TgsGP* PCR
5. Dal069	[246]	*T. b. gambiense*	Human; Côte d'Ivoire; 1981	+	+
12. Biyamina	[248]	*T. b. gambiense*	Human; Sudan; 1982	+	-
20. Katerema 311	[247]	*T. b. brucei*	Cattle; Uganda; 1990	-	+
33. UGH	[247]	*T. b. rhodesiense*	Human; Uganda ; 1988	+	+
62. Muraz 15 IM47[a]	[248]	*T. b. gambiense*	Human; Burkina Faso;1980	-	-

[a] LAMP *PfrA* negative, so may be inadequate quantity/quality DNA in this extraction for molecular analysis

5.4.2 *T. b. gambiense* specific LAMP reaction

Excluding the LAMP *PfrA* negative *Trypanozoon* samples leaves 83 samples, of which 68 were positive when the assay was performed in a real time turbidimeter. The average time to turbidity for the positive samples was 1 h 15 min 38 s. The average time to turbidity for true positives (defined by previous published identity of the same stocks) was 1 h 14 min 41 s, while the average time to turbidity for false positives was 1 h 15 min 47 s. When the reaction was performed in a heating block and the result assessed by gel electrophoresis 21 fewer samples were positive. Of the three LAMP *PfrA* negative samples, 56 and 72 were negative for LAMP TBG1 by both methods, whereas 62 was negative when LAMP TBG1 was assessed by gel, but positive when assessed with the real time turbidimeter.

5.4.3 Agreement between real time turbidimetry and gel electrophoresis for LAMP TBG1

A two by two table quantifying the agreement and disagreement between real time turbidimetry and gel electrophoresis results is shown below (Table 5.3). From this table Cohen's kappa statistic was calculated. Poor agreement [199] ($\kappa = 0.184$; 95 % CI -0.043 – 0.411) was seen between the gel and turbidimetric assessed reactions. The prevalence index was 0.386 and the bias index was 0.253. The maximum attributable kappa was 0.447. The majority of the discordant results are for reactions which were positive by turbidity, but not by gel electorphoresis.

Table 5.3. Two by two table summarising the agreement between real time turbidimetry and gel electrophoresis for LAMP TBG1

	Gel		
Turbidimeter	+	-	**Total**
+	42	26	68
-	5	10	15
Total	47	36	83

5.4.4 Detection of *T. b. gambiense* by LAMP TBG1

The LAMP TBG1 assay for the detection of *T. b. gambiense* (Groups 1 and 2 according to previous identification of these stocks in the literature) was assessed with gel electrophoretic read out. By gel, LAMP TBG1 showed less agreement than would be expected by chance ($\kappa = -0.098$; 95 % CI -0.290 – 0.094) with the published identity of the samples and was less than 50 % sensitive and specific. Secondly, when the outcome was assessed by real time turbidimetry the agreement was poor ($\kappa = 0.009$; 95 % CI -0.128 – 0.145). Sensitivity was improved to 81.82 % with the longer run time, but with specificity reduced to 20.83 % (Tables 5.4 and

5.5). The confidence intervals are particularly wide for the sensitivity estimates, since so few *T. b. gambiense* samples were available.

Table 5.4. Two by two table summarising the agreement between LAMP TBG1 and published identity as *T. b. gambiense* (Group 1 and Group 2)

	LAMP TBG1					
	Gel electrophoresis			Real time turbidimetry		
Published identity	+	-	**Total**	+	-	**Total**
T. b. gambiense	4	7	11	9	2	11
Not *T. b. gambiense*	43	29	72	57	15	72
Total	47	36	83	66	17	83

Table 5.5. LAMP TBG1 compared to published *T. b. gambiense* identity (Group 1 and Group 2)

	LAMP TBG1	
	Gel electrophoresis	**Real time turbidimetry**
Kappa (95 % CI)	- 0.098 (-0.290 – 0.094)	0.009 (-0.128 – 0.145)
Sensitivity (95 % CI)	36.36 % (14.98 – 64.81 %)	81.82 % (51.15 – 96.01 %)
Specificity (95 % CI)	40.28 % (29.71 – 51.83 %)	20.83 % (12.94 – 31.68 %)
NPV (95 % CI)	80.56 % (64.67 – 90.55 %)	88.24 % (64.41 – 97.97 %)
PPV (95 % CI)	8.51 % (2.83 – 20.46 %)	13.64 % (7.12 – 24.15 %)

To aid interpretation of kappa the prevalence and bias indices and the maximum attributable kappa were also calculated (Table 5.6).

Table 5.6. Prevalence and bias indices, as well as the maximum attributable kappa for the data in Tables 5.4 and 5.5.

	LAMP TBG1	
	Gel electrophoresis	**Real time turbidimetry**
κ_{max}	0.210	0.076
Prevalence index	0.301	0.072
Bias index	0.434	0.663

5.4.5 Comparing LAMP TBG1 and *TgsGP* PCR.

LAMP TBG1 showed less agreement than would be expected by chance with the standard PCR method for the detection of *T. b. gambiense*, regardless of the method used to determine the output of the reaction (gel electrophoresis, $\kappa = -0.049$; real time turbidimetry, $\kappa = -0.044$). When assessed by gel electrophoresis the LAMP assay was less than 50 % sensitive and specific compared to the PCR. When the reaction was run for longer in the real time turbidimeter, sensitivity was improved to 66.67 %, but specificity fell to 16.22 % (Tables 5.7 and 5.8). Confidence intervals are particularly wide for the sensitivity estimates, since so few *T. b. gambiense* samples were available.

Table 5.7. Agreement between the LAMP TBG1 and *TgsGP* PCR assays

		LAMP TBG1					
		Gel electrophoresis			Real time turbidimetry		
		+	-	Total	+	-	Total
***TgsGP* PCR**	+	4	5	9	6	3	9
	-	43	31	74	62	12	74
	Total	47	36	83	68	15	83

Table 5.8. Summary statistics comparing LAMP TBG1 with *TgsGP* PCR

	LAMP TBG1	
	Gel electrophoresis	**Real time turbidimetry**
Kappa (95 % CI)	-0.048 (-0.240 – 0.145)	-0.044 (-0.162 – 0.074)
Sensitivity (95 % CI)	44.44 % (18.84 – 73.37 %)	66.67 % (35.09 – 88.27 %)
Specificity (95 % CI)	41.89 % (31.32 – 53.27 %)	16.22 % (9.37 – 26.40 %)
NPV (95 % CI)	86.11 % (70.87 – 94.39 %)	80.00 % (54.05 – 93.70 %)
PPV (95 % CI)	8.51 % (2.83 – 20.46 %)	8.82 % (3.78 – 18.27 %)

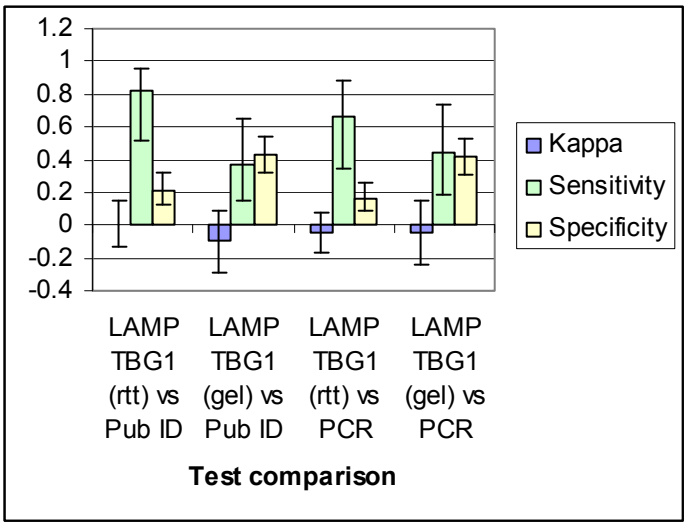
To aid interpretation of kappa the prevalence and bias indices and the maximum attributable kappa were also calculated (Table 5.9).

Table 5.9. Prevalance and bias indices, as well as the maximum attributable kappa for the data in Tables 5.7 and 5.8.

	LAMP TBG1	
	Gel electrophoresis	**Real time turbidimetry**
κ_{max}	0.170	0.052
Prevalence index	0.325	0.566
Bias index	0.458	0.675

Figure 5.1 shows kappa, sensitivity and specificity (with their respective 95 % confidence intervals) for all the comparisons made above.

Figure 5.1. Kappa, sensitivity and specificity estimates, with 95 % CI for all comparisons made between LAMP TBG1 and the reference methods



LAMP TBG1 (rtt) was performed using the real time turbidimeter; LAMP TBG1 (gel) was incubated in a thermocycler with endpoint detection via gel electrophoresis and UV illumination; pub ID is the identity of these samples according to previous analyses recorded in the literature and PCR is *TgsGP* PCR

5.4.6 Sequence alignment of primers to the target region

The LAMP TBG1 primers were not specific for *T. b. gambiense*, whether compared to the previously published identity of these stocks, or to the standard PCR method for the detection of *T. b. gambiense* (the *TgsGP* PCR, whose use is restricted to identification of Group 1 *T. b. gambiense*). The LAMP TBG1 primer sequences were aligned to the 5.8S rRNA gene from four *T. b. brucei* and four *T. b. gambiense* sequences in order to investigate this poor specificity. Several interesting observations were made. Firstly, the primers do not span the *T. b. gambiense* specific C_3A insertion. Secondly, the primers do not show unique and perfect consensus with *T. b. gambiense*. Thirdly, there is intraspecific variation within the primer binding sites for *T. b. gambiense* and *T. b. brucei*. These observations are discussed in more detail below. Tables 5.10 and 5.11 summarise the sequence mismatch between the

primers and these sequences. The primer binding sites on each of the *T. b. brucei* and *T. b. gambiense* ITS1 – 5.8S – ITS2 gene sequences are shown in Appendix 13.

5.4.6.1 The FIP primer

The F2 section of the FIP primer does not show any sequence mismatch with the eight sequenced *T. b. brucei* and *T. b. gambiense* isolates. The F1c section aligns perfectly to all of the *T. b. gambiense* sequences and one of the four *T. b. brucei* sequences, but shows slight mismatch with three of four *T. b. brucei* sequences. This suggests that the FIP primer will show limited specificity to *T. b. gambiense*.

5.4.6.2 The BIP primer

The B2 section of the BIP primer shows similar amounts of sequence mismatch for *T. b. brucei* and *T. b. gambiense* (it is not a perfect match for any of the sequences except the single *T. b. gambiense* isolate it was designed against). The B1c section matches three of four *T. b. brucei* sequences and two of four *T. b. gambiense* sequences (including the one it was designed to). The BIP primer does not show specificity for *T. b. gambiense*.

5.4.6.3 The F3 primer

The F3 primer sequence matches all *T. b. brucei* and *T. b. gambiense* isolates sequenced here. It does not provide specificity for *T. b. gambiense*.

5.4.6.4 The B3 primer

B3 primer shows similar sequence mismatch for *T. b. brucei* and *T. b. gambiense* (it is not a perfect match for any of the sequences except the one *T. b. gambiense* isolate it was designed to). It is unlikely that it could be specific for *T. b. gambiense*.

Table 5.10. A summary of the differences between the binding sites for the TBG1 LAMP primers for *T. b. brucei* isolates (see also Appendix 13)

	Primer					
	F3	**F2**	**F1c**	**B1c**	**B2**	**B3**
AF306770	No difference	No difference	2bp insertion, 2bp substitution	3 1bp substitutions	1bp deletion, 3 1bp substitutions	Very different; possibly 1bp deletion and 5 1bp substitutions
AF306771	No difference	No difference	1 2bp insertion	No difference	4 1bp substitutions	Different in middle; possibly 1bp deletion and 2 1bp substitutions
AF306772	No difference	No difference	No difference	No difference	2 1bp substitutions	Different in middle; possibly 1bp deletion and 2 1bp substitutions
AF306773	No difference	No difference	1bp substitution	No difference	1bp insertion. 2, 1bp substitutions	Different in middle; 1bp deletion,1bp substitution

Table 5.11. A summary of the differences between the binding sites for the TBG1 LAMP primers for *T. b. gambiense* isolates (see also Appendix 13)

	Primer					
	F3	**F2**	**F1c**	**B1c**	**B2**	**B3**
AF306774	No difference	No difference	No difference	4 1bp substitutions	4 1bp substitutions	Different in middle; possibly 1bp deletion and 2 1bp substitutions
AF306775	No difference	No difference	No difference	No difference	1bp insertion, 1bp substitution	Different in the middle, possibly 3 1bp deletions and 1bp substitution
AF306776	No difference	No difference	No difference	1bp substitution	2 1bp substitutions	Different in the middle; possibly 1bp deletion and 2 1bp substitutions
AF306777	No difference	No difference	No difference	No difference	No difference	No difference

5.5 Discussion

5.5.1 *T. b. gambiense* specific PCR

Several PCR assays have been developed for *T. b. gambiense* [109, 263, 264] of which the *TgsGP* PCR is considered the best [177]. Since the first publication of the assay it has been modified into a more sensitive nested PCR reaction [32]. In the present work it was chosen as the best currently available PCR for the molecular identification of *T. b. gambiense*. Notably though it is only useful for detection of Group 1 T. *b. gambiense*. Although Group 1 isolates do comprise the majority of *T. b. gambiense* the inability to detect Group 2 *T. b. gambiense* is a short coming of this PCR for the present study. A Group 2 *T. b. gambiense* specific PCR is not known to be available.

The *TgsGP* PCR showed very good agreement with published identity as a Group 1 *T. b. gambiense* isolate. One false negative result was seen compared to what was expected based on identification of these samples in the literature. This was the sample known as Biyamina, which was instead noted to be *SRA* positive. This concurs with previous unpublished observations in our laboratory (Kim Picozzi, personal communication). Two false positive results were also seen. These may be due to cross contamination of these samples with *T. b. gambiense* DNA, or contamination of second round PCR reaction tubes with first round PCR product from a *T. b. gambiense* positive sample. The nested PCR increases the opportunities for handling errors to be made by increasing the number of manipulations that need to be performed. This is a disadvantage that must be offset against the improvement in sensitivity that is obtained via the nested approach. It is also possible, but less likely, that these isolates (a human isolate from South East Uganda, and a cattle isolate) actually contained *T. b. gambiense* parasites at the time of collection.

Despite these discrepancies, given the good agreement between the observed *TgsGP* PCR result and the identity of these isolates as described in the literature, *TgsGP*

PCR was considered a reasonable molecular test against which to validate the LAMP TBG1.

5.5.2 Real time turbidimetry for LAMP TBG1

LAMP assays can be performed in a heating block, thermocycler or water bath. Positive amplification reactions can be visualised by UV illumination after gel electrophoresis of the reaction products. This approach gives rise to a ladder of bands, which may appear as a more degenerate smear if the electrophoresis is ran more quickly, or through a less stringent gel. Alternatively LAMP reactions can be performed in a real time turbidimeter, typically for 90 minutes, after which the time taken to reach a pre determined turbidity threshold is recorded. If turbidity is noted the reaction is considered positive. Real time turbidimetry allows quantitative objective assessment of turbidity. It is often favoured by researchers developing LAMP reactions since the effect of any changes to primer design or reaction composition on the efficiency of the reaction can be quantified. Furthermore the reaction tube does not need to be opened. A closed tube system is preferred so that a laboratory does not become contaminated with LAMP products, which might compromise future work.

In this study, an additional 21 positive results were seen when the LAMP TBG1 assay was performed using a real time turbidimeter, rather than a heating block (when the reaction was performed in a heating block results were visualised by gel electrophoresis and UV illumination). Agreement between these two different methods of performing the same assay was poor, most likely as the reaction was incubated for longer in the real time turbidimeter. Indeed, only one sample reached the turbidity threshold in less than one hour. This could also be because the turbidity threshold which was chosen was inappropriate. However, spurious turbidity must not be ruled out. Differences could also be due to actual amplification differences between the two repeats of the reactions, rather than differences in the detection

methods per se. The disagreement seen here serves as a warning for development and validation studies for other LAMP primer sets. Firstly, are all, or certain, LAMP reactions intrinsically unreliable, such that two screens of any sample set will never produce congruent results? (addressed in Chapter 7). Secondly, is the disagreement seen between the gel and turbidimeter results here a problem for other LAMP primer sets? Thirdly, is the disagreement here due to poor calibration of the real time turbidimetry threshold? In this study the choice of the turbidity threshold was arbitrarily chosen as a typical value used by other researchers for development of LAMP assays. Perhaps a threshold ought to be specifically chosen for a given LAMP assay by comparison to gel electrophoresis results?

5.5.3 Validation of LAMP TBG1

LAMP TBG1 analysed by gel showed less agreement than would be expected by chance with published identity as *T. b. gambiense*. This comparison was made against published identity as either Group 1 or 2 *T. b. gambiense*, since LAMP TBG1 was validated for identification of both groups in the original publication. LAMP TBG1 by gel also showed less agreement than would be expected by chance with results from the *TgsGP* PCR which is specific for *T. b. gambiense* Group 1.

When LAMP TBG1 results from the real time turbidimeter assays were compared to published identity as *T. b. gambiense* (Group 1 and 2) there was almost no agreement beyond what would be expected by chance. The sensitivity was improved compared to the gel electrophoresis results, but the specificity was markedly worse. This reflects the general observation that more samples are positive by real time turbidimeter.

When LAMP TBG1 results from the real time turbidimeter were compared to the *TgsGP* PCR results there was less agreement than would be expected by chance, sensitivity was better than that seen by gel, but specificity was worse. This reflects

the observation that more samples are positive, in a non-specific manner, by real time turbidimetry.

Although LAMP TBG1 was not described as being specific for Group 2 *T. b. gambiense*, the specific sequence of the target gene used during the primer design process was from a Group 2 *T. b. gambiense* isolate. In the analyses LAMP TBG1 was compared to both Group 1 and 2 *T. b. gambiense* isolates since the assay was originally published for use with both of these groups. However, given that primers were designed using a sequence from a Group 2 isolate it is not surprising that marginally better agreement was seen when the assay was compared to all *T. b. gambiense*, as identified in the literature, as opposed to *TgsGP* PCR positives as defined in this study.

A closer look at the binding sites of the LAMP TBG1 primers for four *T. b. brucei* and three Group 1 *T. b. gambiense* sequences as well as the Group 2 *T. b. gambiense* sequence used for primer design was enlightening. LAMP TBG1 primers did not account for the C_3A insertion previously reported to be unique and specific for *T. b. gambiense* in this region. The FIP primer shows limited specificity for *T. b. gambiense* for these sequenced isolates. None of the other primers look to be *T. b. gambiense* specific, however one LAMP primer may be sufficient to confer specificity on a whole assay. The intra subspecies variability in the primer binding sites is also a concern since it might reduce the sensitivity of these primers.

5.5.4 Conclusions

The results described here do not support the use of the LAMP TBG1 assay for *T. b. gambiense* specific molecular detection and identification. This work highlights areas for concern when performing LAMP assays using real time turbidimetry rather than incubation followed by gel electrophoresis and UV visualisation of LAMP products.

6 Chapter 6. Design and evaluation of a novel LAMP protocol for the specific detection of Group 1 *Trypanosoma brucei gambiense*

6.1 Introduction

In Chapter 5 a LAMP assay for *T. b. gambiense* (LAMP TBG1) [141] was evaluated using 86 trypanosome DNA samples (including nine from Group 1 *T. b. gambiense* isolates). Regardless of the method used for assay incubation and read out, and regardless of whether results were compared to the previously published identity or *TgsGP* PCR results for these DNA samples, LAMP TBG1 showed poor agreement (Cohen's kappa statistic (κ) = 0 – 0.2) or less agreement than would be expected by chance ($\kappa < 0$) [199]. Hence, LAMP TBG1 protocol does not provide an accurate diagnostic tool for *T. b. gambiense* and there remains a requirement for the design of a sensitive and specific LAMP assay for *T. b. gambiense*.

The broad spectrum LAMP RIME [121] and LAMP *PfrA* [140] assays, which detect all members of the subgenus *Trypanozoon*, could be used for the diagnosis of *T. b. gambiense*, but fail to differentiate *T. b. gambiense* from *T. b. rhodesiense*. Clinically this would be problematic if a patient had travelled within endemic foci of both diseases, or if the foci of these two separate diseases merge. Since the convergence of *T. b. gambiense* and *T. b. rhodesiense* endemic foci in Uganda is becoming increasingly likely, the need for a discriminatory diagnostic test for these two subspecies is becoming increasingly urgent [32]. Generic *Trypanozoon* diagnostics might also detect DNA from abortive or transient *T. b. brucei* infections [39, 91, 92]. Separate concerns regarding the sensitivity and specificity of currently available LAMP assays for *Trypanozoon*s are discussed in Chapter 3.

Here is a description of the design, development and validation of a novel LAMP assay for Group 1 *T. b. gambiense*. The *TgsGP* gene was selected as the target for

this novel assay. *TgsGP* was discovered in 2001 and encodes a *T. b. gambiense* specific glycoprotein [25]. It is well conserved in Group 1 *T. b. gambiense*, regardless of geographic origin and PCR assays have been developed (see Chapter 1, Section 1.7.3). Recall that since *TgsGP* like genes have been identified in other *T. brucei* s.l. isolates [25, 109, 265] with consensus to the 5' end of *TgsGP*, *TgsGP* specific primers should be targeted to the 3' end, where similarity to possible ancestral/ related genes is lost [109, 177].

Here LAMP for *TgsGP* was validated using 86 trypanosome samples of DNA that were previously used to evaluate the LAMP TBG1 assay. The absolute sensitivity of this novel assay is also determined, and simple endpoint detection by reaction tube turbidity is established. The reliability of the assay is analysed and described in Chapter 7.

6.2 Aims

1. To design LAMP primers targeted to the *TgsGP* PCR gene.
2. To develop a LAMP assay for amplification of *T. b. gambiense*.
3. To validate the LAMP assay on a large set of *Trypanozoon* DNA samples.

6.3 Study outline

Firstly, a novel LAMP assay was designed to be specific for *T. b. gambiense* and was trialled with a small sub set of *T. b. gambiense* and non *T. b. gambiense* DNAs using a typical LAMP reaction mix, over a range of $MgSO_4$ concentrations.

Secondly the assay was adapted for use in the real time turbidimeter.

Thridly, it was applied to the 86 DNA samples, once using a real time turbidimeter and once in a heating block followed by gel electrophoresis and turbidity assessment by eye. These analyses were in addition to all previous molecular characterisations performed on these samples.

Cross tabulations were used to calculate sensitivity, specificity, negative and positive predictive values and Cohen's kappa statistic, as well as 95 % confidence intervals, the prevalence and bias indices, and the maximum attributable kappa to aid the interpretation of kappa. These calculations were performed to compare:

(i) Published identity as Group 1 *T. b. gambiense* against
   a. LAMP *TgsGP* (assessed three times by gel electrophoresis)
   b. LAMP *TgsGP* (assessed once by real time turbidimetry);

(ii) *TgsGP* PCR against
   c. LAMP *TgsGP* (assessed three times by gel electrophoresis)
   d. LAMP *TgsGP* (assessed once by real time turbidimetry);

(iii) LAMP *TgsGP* (assessed by gel electrophoresis) against LAMP *TgsGP* (assessed by real time turbidimetry)

Fourthly, the absolute sensitivity of LAMP was established in comparison to PCR.

6.4 Results

6.4.1 Molecular assays

The results of the PCR assays and the LAMP *PfrA* for all 86 samples are tabulated in Appendix 12 and described in detail in Chapters 4 and 5. Key points to note here are that (i) three samples (56, 62 and 72) were excluded from all analyses because they were LAMP *PfrA* negative, despite being TBR PCR positive and (ii) the *TgsGP* PCR showed very good (although imperfect) agreement ($\kappa = 0.803$) with identity as Group 1 *T. b. gambiense* according to the literature.

6.4.2 Initial trial and adaptation for use with a real time turbidimeter

In the initial trial, positive LAMP endpoints were seen for *TgsGP* PCR positive but not *TgsGP* PCR negative samples at 4 mM, 6 mM and 8 mM $MgSO_4$. With 2 mM $MgSO_4$ positive LAMP amplification was not observed. Specific LAMP positive endpoints were also seen when the reaction was performed in the real time turbidimeter. In the real time turbidimeter the time to threshold turbidity (Tt) was much quicker when 1.4 mM dNTPs were included (Table 6.1).

Table 6.1. Effect of dNTP concentration on the efficiency of the LAMP *TgsGP* reaction

Sample	Sub-species	Tt	
		0.2 mM dNTPs	**1.4 mM dNTPs**
5.	*T. b. gambiense*	73 min 54 s	37 min 30 s
17.	*T. b. gambiense*	78 min 0 s	40 min 0 s
1.	*T. b. brucei*	No amplification	No amplification
3.	*T. b. rhodesiense*	No amplification	No amplification

6.4.3 Application of the *TgsGP* LAMP assay to 86 trypanosome DNA samples

Firstly, the assay was applied to the 86 samples using a real time turbidimeter (see full results in Appendix 16, Table 1). In summary, nine *TgsGP* samples reached the turbidity threshold in 90 minutes, of which six were true positive (positive by both *TgsGP* PCR and in the literature) and three were false positive (negative by both *TgsGP* PCR and in the literature). The average time to reach the turbidity threshold was 60 min 31 s. For the six true positive results the average time to turbidity was 51 min 6 s, whereas for the three false positive results the average time to turbidity

was 79 min 6 s. There is a marked difference in the time to turbidity between true and false positive samples.

Secondly, the same assay was applied to the same samples using a thermocycler. Samples were incubated for 60 (rather than 90) minutes, and endpoint detection was performed by standard gel electrophoresis with UV illumination and by visualisation of endpoint turbidity. The second method was performed three times for the full sample set (full results are shown in Appendix 16, Table 2).

6.4.3.1 Accuracy of the TgsGP LAMP assay

a) Incubation and readout using a real time turbidimeter

When a real time turbidimeter was used for incubation and readout, the LAMP *TgsGP* showed moderate agreement with the *TgsGP* PCR assay (κ = 0.504; 95 % CI 0.177 – 0.832) and good agreement with published identity as a Group 1 *T. b. gambiense* isolate (κ = 0.672; 95 % CI 0.394 – 0.951). Table 6.2 presents a two by two comparison of the real time turbidimeter results for the *TgsGP* LAMP versus *TgsGP* PCR and published identity as Group 1 *T. b. gambiense*. Table 6.4 shows the summary statistics for these comparisons.

Table 6.2. Agreement of *TgsGP* LAMP (by real time turbidimeter) with (i) published identity as Group 1 *T. b. gambiense* and (ii) *TgsGP* PCR

	Published identity as Group 1 *T. b. gambiense*			***TgsGP* PCR**		
LAMP *TgsGP*	+	-	**Total**	+	-	**Total**
+	6	3	9	5	4	9
-	2	72	74	4	70	74
Total	8	75	83	9	74	83

Table 6.3. Summary statistics comparing LAMP *TgsGP* (by real time turbidimeter) with (i) published identity as Group 1 *T. b. gambiense* and (ii) *TgsGP* PCR

	Published identity as Group 1 *T. b. gambiense*	***TgsGP* PCR**
Kappa	0.672	0.504
(95 % CI)	(0.394 – 0.951)	(0.177 – 0.832)
Sensitivity	75.00 %	55.56 %
(95 % CI)	(40.09 – 93.69 %)	(26.63 – 81.16 %)
Specificity	96.00 %	94.59 %
(95 % CI)	(88.42 – 99.10 %)	(86.50 – 98.28 %)
NPV	97.30 %	94.59 %
(95 % CI)	(90.10 – 99.82 %)	(86.50 – 98.28 %)
PPV	66.67 %	55.56 %
(95 % CI)	(35.09 – 88.27 %)	(26.63 – 81.16 %)

To aid interpretation of kappa the prevalence and bias indices and the maximum attributable kappa were also calculated (Table 6.4).

Table 6.4. Prevalence and bias indices, as well as the maximum attributable kappa for the data in Tables 6.2 and 6.3.

	Published identity as Group 1 *T. b. gambiense*	***TgsGP* PCR**
κ_{max}	0.934	1
Prevalence index	0.795	0.783
Bias index	0.012	0

b) Incubation followed by gel electrophoresis

The LAMP *TgsGP* reaction was performed in triplicate, in a thermocycler followed by endpoint turbidity and gel electrophoretic assessment. An assessment of the reliability of this assay, using these results, is addressed in Chapter 7 and the use of turbidity as a simple method for readout, using these results, is discussed in Chapter 8. Here, analysis is focussed on the accuracy of the LAMP *TgsGP* assay.

When the *TgsGP* LAMP, with the results read by gel electrophoresis, was compared to the published identity of these isolates, the agreement, as measured by Cohen's kappa statistic, ranged from 0.467 to 0.862. The two by two comparisons and summary statistics are shown in Tables 6.5 and 6.6 respectively.

Table 6.5. *TgsGP* LAMP, incubated in a thermocycler and assessed by gel electrophoresis, applied three times to the sample set, versus published identity as Group 1 *T. b. gambiense*

	Replicate of LAMP *TgsGP*								
	1			**2**			**3**		
Published identity	**+**	**-**	**Total**	**+**	**-**	**Total**	**+**	**-**	**Total**
Group 1 *T. b. gambiense*	7	1	8	5	3	8	7	1	8
Non- Group 1 *T. b. gambiense*	1	74	75	6	69	75	8	67	75
Total	8	75	83	11	72	83	15	68	83

Table 6.6. Summary statistics comparing the three LAMP *TgsGP* replicates (incubated in a thermocycler and assessed by gel electrophoresis) versus published identity as Group 1 *T. b. gambiense*

	Replicate of LAMP *TgsGP*		
	1	**2**	**3**
Kappa (95 % CI)	0.862 (0.672 – 1.051)	0.467 (0.138 – 0.796)	0.552 (0.276 – 0.829)
Sensitivity (95 % CI)	87.50 % (50.78 – 99.89 %)	62.50 % (30.38 – 86.51 %)	87.50 % (50.78 – 99.89 %)
Specificity (95 % CI)	98.67 % (92.13 – 99.99 %)	92.00 % (83.32 – 96.59 %)	89.33 % (80.10 – 94.74 %)
NPV (95 % CI)	100 % (94.09 – 100 %)	95.83 % (87.97 – 99.06 %)	98.53 % (91.37 – 99.99 %)
PPV (95 % CI)	87.50 % (50.78 – 99.89 %)	45.45 % (21.25 – 72.01 %)	46.67 % (24.80 – 69.89 %)

To aid interpretation of kappa the prevalence and bias indices and the maximum attributable kappa were also calculated (Table 6.7).

Table 6.7. Prevalence and bias indices, as well as the maximum attributable kappa for the data in Tables 6.5 and 6.6.

	Replicate of LAMP *TgsGP*		
	1	**2**	**3**
κ_{max}	1	0.822	0.652
Prevalence index	0.807	0.771	0.723
Bias index	0	0.036	0.084

When the *TgsGP* LAMP, with the results read by gel electrophoresis, was compared to the *TgsGP* PCR results for these samples, the agreement, as measured by Cohen's kappa statistic, ranged from 0.546 to 0.934. The two by two comparisons and summary statistics are shown in Tables 6.8 and 6.9 respectively.

Table 6.8. *TgsGP* LAMP, incubated in a thermocycler and assessed by gel electrophoresis, applied three times to the sample set, versus *TgsGP* PCR.

	Replicate of LAMP *TgsGP*								
	1			**2**			**3**		
Published identity	**+**	**-**	**Total**	**+**	**-**	**Total**	**+**	**-**	**Total**
Group 1 *T. b. gambiense*	8	1	9	6	3	9	8	1	9
Non- Group 1 *T. b. gambiense*	0	74	74	5	69	74	7	67	74
Total	8	75	83	11	72	83	15	68	83

Table 6.9. Summary statistics comparing the three LAMP *TgsGP* replicates (incubated in a thermocycler and assessed by gel electrophoresis) with *TgsGP* PCR

	Replicate of LAMP *TgsGP*		
	1	**2**	**3**
Kappa (95 % CI)	0.934 (0.807 – 1.062)	0.546 (0.247 – 0.845)	0.614 (0.360 – 0.868)
Sensitivity (95 % CI)	88.89 % (54.33 – 99.99 %)	66.67 % (35.09 – 88.27 %)	88.89 % (54.33 – 99.99 %)
Specificity (95 % CI)	100 % (94.09 – 100 %)	93.24 % (84.79 – 97.43 %)	90.54 % (81.46 – 95.62 %)
NPV (95 % CI)	98.67 % (92.13 – 99.99 %)	95.83 % (87.97 – 99.06 %)	98.53 % (91.37 – 99.99 %)
PPV (95 % CI)	100 % (62.78 – 100 %)	54.55 % (27.99 – 78.75 %)	53.33 % (30.11 – 75.20 %)

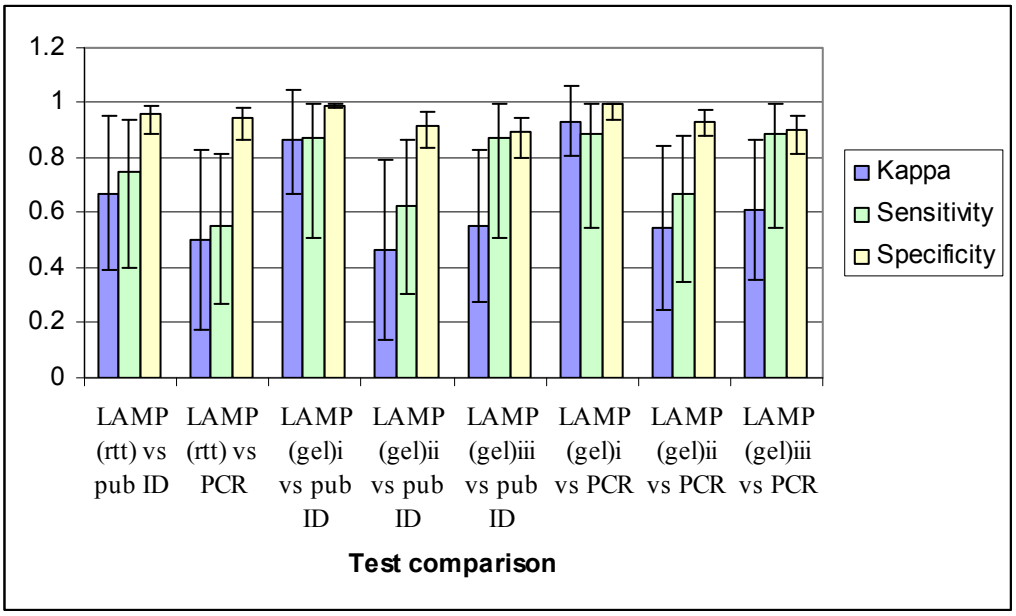
To aid interpretation of kappa the prevalence and bias indices and the maximum attributable kappa were also calculated (Table 6.10).

Table 6.10. Prevalence and bias indices, as well as the maximum attributable kappa for the data in Tables 6.9 and 6.10.

	Replicate of LAMP *TgsGP*		
	1	**2**	**3**
κ_{max}	0.934	0.886	0.711
Prevalence index	0.795	0.759	0.711
Bias index	0.012	0.024	0.072

Overall the best performance of the *TgsGP* PCR assay was the first screen of the sample set in the thermocycler (as assessed by gel electrophoresis) and compared to the *TgsGP* PCR, where the two assays showed very good agreement ($\kappa = 0.934$), with the LAMP test being 89.89 % sensitive and 100 % specific as compared to the PCR (see Figure 6.1).

Figure 6.1. Kappa, sensitivity and specificity estimates, with 95 % confidence intervals for all comparisons made between LAMP *TgsGP* and the reference methods



LAMP (rtt) is LAMP *TgsGP* performed using the real time turbidimeter; LAMP (gel) is LAMP *TgsGP* incubated in a thermocycler with endpoint detection via gel electrophoresis and UV illumination; pub ID is the identity of these samples according to previous analyses recorded in the literature and PCR is *TgsGP* PCR.

6.4.4 Agreement between real time turbidimetry and gel electrophoresis for LAMP *TgsGP*

A two by two table quantifying the agreement and disagreement between real time turbidimetry and gel electrophoresis results (for the first of the three screens only) is shown in Table 6.11. From this table Cohen's kappa statistic was calculated and moderate agreement [199] ($\kappa = 0.541$; 95 % CI 0.216 – 0.867) was observed between the gel and turbidimeter assessed reactions. The prevalence index was 0.795 and the bias index was 0.012. Given that the prevalence index is high the kappa might be

artificially decreased. The maximum attributable kappa was 0.934 and the majority of the discordant results were almost exactly evenly distributed.

Table 6.11. Two by two table summarising the agreement between real time turbidimetry and gel electrophoresis for LAMP *TgsGP*

	Gel		
Turbidimeter	+	-	**Total**
+	5	4	9
-	3	71	74
Total	8	75	83

6.4.5 Absolute sensitivity of LAMP versus PCR

TgsGP PCR was more sensitive than LAMP *TgsGP*. This is shown in Table 6.12 below, which summarises the results when two fold and ten fold dilution series were made from three samples, each with a distinct starting DNA concentration. In all instances the LAMP reaction amplified a 1 in 10, but not a 1 in 100, dilution, whereas the PCR reaction detected *T. b. gambiense* DNA up to, but not beyond, a 1 in 100,000 dilution.

Table 6.12. Detection limits of the *TgsGP* PCR and LAMP assays

Sample	Starting concentration of DNA	*TgsGP* PCR on dilution series		LAMP *TgsGP* on dilution series	
		2 fold	10 fold	2 fold	10 fold
5	27.3 ng µl-1	No limit	1 in 100,000	1 in 64	1 in 10
18	27.4 ng µl-1	No limit	1 in 100,000	1 in 32	1 in 10
58	14.7 ng µl-1	No limit	1 in 10,000	1 in 8	1 in 10

6.5 Discussion

Here a novel LAMP assay for the detection and identification of Group 1 *T. b. gambiense* has been developed and evaluated. The *TgsGP* gene was selected as the target for this assay because it is a well accepted diagnostic marker gene for Group 1 *T. b. gambiense* [109, 177]. Online LAMP primer design software was used to design LAMP primers for *TgsGP* that specifically targeted the 3' end of the gene.

The novel LAMP primers were initially tested on two *T. b. gambiense* Group 1 DNA samples which were both positive by the *TgsGP* PCR assay, and three non-*T. b. gambiense* samples, including two *T. b. brucei* and one *T. b. rhodesiense*. The same reaction conditions were selected that had been used for the LAMP TBG1 assay (63 ˚C for 1 h followed by 2 min at 80 ˚C). At first, the composition of the reaction mix was the same as the published LAMP RIME [121] and *SRA1* [122] reactions (see Chapter 2). However, this was modified with supplementary $MgSO_4$, and additional dNTPs, so that these reagents were present in the same concentrations as in the published LAMP *PfrA* and TBG1 reactions [140, 141]. When the real time turbidimeter was used the reaction buffer was chosen to contain Tween-20, otherwise a reaction buffer containing Triton X-100 was used. Under the modified conditions, *T. b. gambiense* specific LAMP amplification was seen for this subset of samples

using the real time turbidimeter and by heating block and gel electrophoresis, and the assay was applied to the total set of samples.

The LAMP *TgsGP* assay was applied to all samples, firstly using a real time turbidimeter, and secondly using a thermocycler. In the real time turbidimeter the samples were incubated for 90 min, without a termination step, and the result was counted as positive if turbidity surpassed the threshold value of 0.1 within 90 min. With the thermocycler the reaction was incubated for 1 h, followed by a 2 min termination step. The reaction was counted as positive if a ladder/smear of DNA could be seen upon UV illumination of the products run through an agarose gel. The turbidity of the reaction at the end time was also assessed by eye. With the thermocycler the full set of samples was screened three times.

With the real time turbidimeter, the average time to the reach the turbidity threshold, was considerably slower for the false positive reactions, than for the true positives. The turbidity threshold that was used was not calibrated for this reaction but was simply chosen as a value typically used when developing LAMP reactions. If incubation had been limited to one hour these false positives could be properly interpreted as negative results without affecting any of the true positives. Alternatively, selection of a different turbidity threshold might also have eliminated the false positives seen here. This demonstrates the importance of calibration of the turbidity against gel electrophoresis, when developing any novel LAMP assay. It would be useful to apply the LAMP *TgsGP* assay using a dilution series of *TgsGP* positive and negative samples with different turbidity thresholds and for different time points, in order to determine the best endpoint for the assay.

The average time to reach the turbidity threshold was slower, even when considering the true positive results alone, than for the LAMP *PfrA* reaction. This suggests that the LAMP *TgsGP* reaction is less efficient. Several approaches for improving the

efficiency of the reaction could be made; firstly the primers themselves could undergo slight adjustments so that they bind in slightly different positions. Small alterations in the primer-binding site have been shown to have dramatic effects on reaction efficiency during the development of other LAMP assays (Chie Nakajima, pers. com.). Secondly the relative concentrations of the inner, outer and loop primers could be modified. Again, this has been shown to affect reaction efficiency during the development of other LAMP assays (Chie Nakajima, personal communication). Finally, small adjustments in the composition of the reaction mix, or the incubation temperature might improve the efficiency of the reaction. Having demonstrated the potential of this primer set in this work, further improvements could be worthwhile.

With the real time turbidimeter LAMP *TgsGP* showed moderate agreement (κ = 0.501; 95 % CI 0.177 – 0.832) with the *TgsGP* PCR assay but good agreement with published identity as a Group 1 *T. b. gambiense* isolate (κ = 0.672; 95 % CI 0.394 – 0.951). This difference arises from discrepancies between the *TgsGP* PCR results and the published identity of three of the samples. The basis on which a sample was categorised as Group 1 is summarised in Appendix 11 and *TgsGP* PCR results are shown in Appendix 16. Sample 12 which is a Group 1 *T. b. gambiense* according to isoenzyme analyses the literature [248] was negative by the *TgsGP* PCR but positive by the LAMP *TgsGP* (with the turbidimeter). Samples 20 and 33 are *TgsGP* PCR positive, but are not *T. b. gambiense* according to the literature (see Appendix 2). Both are LAMP *TgsGP* negative in the turbidimeter. The wide confidence intervals of both kappa estimates overlap considerably and one cannot confidently conclude that the difference in agreement is significant.

The results were initially very promising when the reaction was performed with the thermocycler, with read out by UV illumination after gel electrophoresis. For the first screen, the LAMP *TgsGP* was 88.89 % sensitive (95 % CI 54.33 – 99.99 %) and 100 % specific (95 % CI 94.09 – 100 %) compared to *TgsGP* PCR. Although the 95 % confidence interval around the sensitivity estimate is wide, owing to the small number of *T. b. gambiense* samples that were available, it remains well above zero so

one can be confident that agreement exceeds that expected by chance alone. Here the LAMP assay appeared more favourable when compared to the *TgsGP* PCR assay rather than the published identity of the isolates, although the difference was insignificant given the overlap in the confidence intervals. Again the differences are due to samples 12, 20 and 33, as summarised in Table 6.13 below. The different results seen for these three samples explain what first appeared to be the strange observation that with the real time turbidimeter the *TgsGP* LAMP assay agreed more strongly with published identity whereas when the reaction was performed using a thermocycler and assessed by gel electrophoresis with UV illumination, the assay agreed more strongly with the *TgsGP* PCR results.

Table 6.13. Discrepant results for samples 12, 20 and 33

Sample	Published identity	Ref	*TgsGP* PCR (this study)	LAMP *TgsGP* - real time turbidimeter	LAMP *TgsGP*- thermocycler and gel electrophoresis (1st repeat)
12	Group 1 *T. b. gambiense*	[248]	-	+	-
20	*T. b. brucei*	[247]	+	-	+
33	*T. b. rhodesiense*	[247]	+	-	-

The results from the first screen in the thermocycler (assessed by gel electrophoresis) were also compared to the results when the assay was performed in the real time turbidimeter. These two data sets showed moderate agreement, which contrasts with the poor agreement seen in Chapter 5 when real time turbidimeter and themocycler results were compared for the LAMP TBG1 assay. Most importantly an excess of false positive results was not observed for LAMP *TgsGP* with the real time turbidimeter. This reinforces the utility of the turbidimeter if an appropriate turbidity

threshold is selected. Here, the selection of an appropriate turbidity threshold was largely serendipitous. The remaining discrepancies are not unexpected given that the repeatability of the assay was imperfect even when performed under identical conditions (see Chapter 7). However, at first glance alone we can see that the agreement over several repeats is far from perfect. This is possibly due to degradation of the DNA samples and the increasing likelihood of cross contamination between samples over time and with repeated use.

LAMP is often promoted as being more sensitive than PCR, however, here the *TgsGP* nested PCR is 1,000 to 10,000 times more sensitive than the LAMP reaction. It is possible that the sensitivity of this LAMP assay might be improved by small adjustments to the reaction composition and conditions.

The use of turbidity as a simple method for LAMP *TgsGP* readout is discussed in more detail in Chapter 8. In short, visual turbidity was less sensitive than gel electrophoresis but was 100 % specific for gel positive endpoints, as assessed by a single, experienced reader. Therefore, turbidity could be useful quick method for reading LAMP results.

In conclusion, novel LAMP primers for *T. b. gambiense* were designed to target the same region of the *TgsGP* gene as the nested *TgsGP* PCR [32] and the results shown here are promising. At best, Cohen's kappa statistic for the agreement between the LAMP and PCR assays was 0.934. The LAMP assay detected 88.89% of the PCR positive samples, and no false positives were seen. Given these strong results the process was repeated in order to assess the reliability of this assay. However, when the assay was repeated both sensitivity and specificity were reduced and the assay was observed to be less than perfectly reliable (see Chapter 7). In addition to considering diagnostic sensitivity it is also important to consider the absolute sensitivity of any molecular diagnostic. LAMP is promoted as being more sensitive

that PCR. However, this novel LAMP assay was consistently less sensitive than the equivalent PCR. In part this may be attributed to the nested nature of the PCR, though it also highlights the possibility to further improve the sensitivity of this promising new LAMP assay. Finally, it is important to note that the evaluation performed here was unfortunately limited by the number of *T. b. gambiense* isolates available. Thus confidence limits around the sensitivity estimates, as well as Cohen's kappa statistic are rather wide.

7 Chapter 7. The reliability of LAMP

7.1 Introduction

In the previous chapters the focus has been upon the validity and accuracy of various published and novel LAMP assays for *Trypanozoon* parasites. A valid test is one that measures what it claims to measure. Accuracy is the extent to which this measurement is correct and is quantified by sensitivity and specificity. However, the value of a test also depends upon its reliability. A reliable test generates the same result over several repeats under the same test conditions [266]. Here, the terms reliability and repeatability are used interchangeably. Reproducibility refers to a similar but subtly different concept. While reliability assessment considers the closeness of test results when the conditions of measurement are constant, reproducibility is a measure of the closeness of test results when the conditions for testing or measurement are changed [267]. Changes may include the operator (inter-observer reproducibility), test site, kit lot or run day, for example.

Neither the repeatability nor the reproducibility of the various published LAMP assays for *Trypanozoon*s, have been well addressed in the scientific literature. Reliability was not addressed during publication of the LAMP *PfrA* [140] assay or the LAMP TBG1 [141] assay (which been shown to be non specific see Chapter 5). In the original LAMP *SRA*1 [122] study the assay was performed in triplicate on dilution series of infected mouse blood when determining the analytical sensitivity of the assay: reliability was not reported to be a problem, but neither was it explicitly quantified. The authors did not report any problems with repeatability for LAMP RIME [121]; the results were identical for dilution series of infected mouse blood when performed in a thermocycler or water bath, and there was total agreement between replicates for clinical samples. However the number of replicates applied was not clear.

Follow up reports, in which evaluation of LAMP *SRA*1 and LAMP RIME diagnostics have been extended to clinical samples, have also focussed upon validity assessment [268]. This is not unusual; Guthmann *et al.* [269] noted a similar bias during early evaluations of rapid diagnostic tests (RDTs) for malaria. Yet, reliability is critical and must be analysed and reported. Referring to rapid diagnostic tests for malaria, it has been noted elsewhere that, '*RDTs must provide consistently accurate tests and be seen to do so.* ' [270].

Therefore, here the reliability of several LAMP assays is explicitly addressed. The assays under consideration are: LAMP RIME [121], LAMP *PfrA* [140], LAMP *SRA*1 [122], SRALAMP_a (see Chapter 4) and LAMP *TgsGP* (see Chapter 6). Reliability is assessed using control DNA samples as well as human and animal blood samples.

Here, the endpoint of all LAMP reactions was assessed by gel electrophoresis and UV illumination. The reproducibility of endpoint detection methods is a separate issue that is addressed in Chapter 8.

7.2 Aims

1. To quantify the reliability of LAMP assays for *Trypanozoon* (LAMP RIME and LAMP *PfrA*), for *T. b. gambiense* (LAMP *TgsGP*) and *T. b. rhodesiense* (LAMP *SRA*1 and SRALAMP_a) on the same DNA extractions used for validity analyses throughout this thesis.
2. To quantify the reliability of LAMP RIME, LAMP *PfrA* in comparison to the TBR PCR on human and cattle blood samples.
3. To quantify the reliability of the two LAMP assays for *T. b. rhodesiense* in comparison to the multiplex *SRA* PCR on human and cattle blood samples.

7.3 Study outline

Firstly LAMP RIME [121] and LAMP *PfrA* [140] for *Trypanozoon*s, LAMP *TgsGP* for *T. b. gambiense* (see Chapter 6) and two LAMP reactions for *T. b. rhodesiense* (LAMP *SRA*1 [122] and SRALAMP1 (see Chapter 4) were performed in triplicate on all 86 control DNA samples. Cohen's kappa statistic was used to compare each repeat screen (i, ii and iii) of the total sample set for each assay against the relevant reference PCR (i.e. LAMP *PfrA* and LAMP RIME were compared to the TBR PCR, while LAMP *SRA*1 and SRALAMP_a were compared to the *SRA* PCR). Cohen's kappa statistic was also used to quantify test-retest agreement for all three combinations of the three screens by each assay (eg. for LAMP RIME repeat i was compared to repeat ii and repeat iii; repeats ii and iii were also compared to one another).

Secondly, the same assays were performed in pentaplicate on a subset of the control DNA samples. The number of samples for which all three (or five) replicates were all positive, or all negative were summed and this was calculated as a percentage of the total number of observations (n = 86) to give the overall percentage agreement. The method of Fleiss, Levin *et al.* [271] was used to quantify the agreement across the five replicates for the subspecies specific LAMP assays (LAMP *SRA*1, SRALAMP_a and LAMP *TgsGP*). Hereon this is referred to as Fleiss' kappa

Mostly, repeats were performed serially and the time lag between replicates is summarised here. LAMP *PfrA* replicates were made over two days. LAMP RIME and SRALAMP_a replicates spanned a three-week time period. The first three replicates of the LAMP *TgsGP* assay were performed within nine days, but the additional two repeats were not performed until one month later. Finally, the longest time lapse between repeats was seen for the LAMP *SRA*1 assay. The first full screen of the 86 samples took place five months in advance of the remaining replicates,

which were performed within a three week period. In between the samples were stored in a refrigerator.

Thirdly, the LAMP assays for *Trypanzoon* and *T. b. rhodesiense* (LAMP RIME, LAMP *SRA1* and SRALAMP_a) were performed in triplicate on Whatman FTA card samples

(i) 52 *T. b. rhodesiense* human patient blood samples, alongside triplicate repeats of the TBR and *SRA* PCR assays (each assay was performed simultaneously within 24 hours of DNA preparation)

(ii) 48 cattle blood samples, as well as triplicate repeats of the TBR and *SRA* PCR assays (the TBR PCR and LAMP RIME assays were performed simultaneously within 24 hours of DNA preparation which was then frozen and thawed once before the SRA PCR, LAMP *SRA1* and SRALAMP_a reactions were performed simulataneously on the DNA samples)

The number of samples for which the three replicates were all positive or all negative was calculated as a percentage of the total number of observations to provide the overall percentage agreement. Cohen's kappa statistic was used to quantify test-retest agreement for all three combinations of the three screens by each assay. The magnitude and range of these kappa values for each assay could be compared to one another. Firstly, calculations were made separately for cattle and human sample results, then, in order to increase sample size and reduce confidence intervals calculations were performed on data pooled for all Whatman FTA card field samples. For the *SRA* PCR kappa was calculated from a three by three contingency table, including 'insufficient DNA present' as the third category, where a *GPI-PLC* band was not seen.

7.4 Results

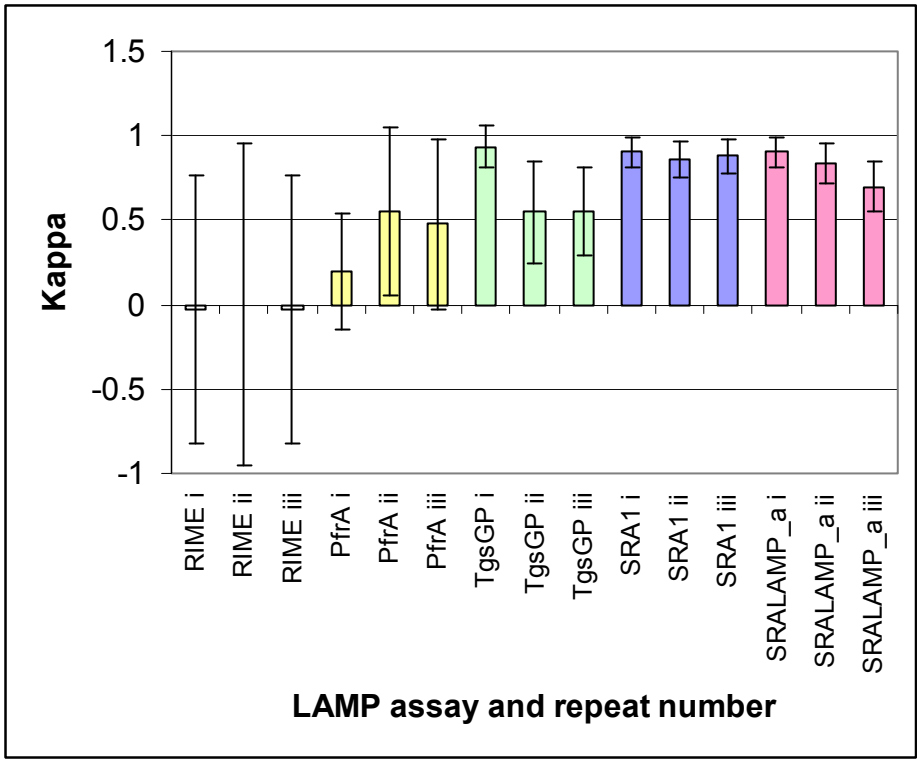
7.4.1 Control DNA samples

The results from the repeated application of the five LAMP assays to the 86 DNA samples can be found alongside the reference PCR results, tabulated in Appendix 17.

7.4.1.1 Agreement between LAMP and the equivalent PCR assays

Having applied LAMP assays to all 86 control DNA samples, in triplicate, the first analysis to be made was a comparison of each repeated application of a particular assay to the appropriate reference PCR (see Figure 7.1 for a graphical results summary). Agreement was quantified by Cohen's kappa statistic. The values of κ_{max}, the prevalence and bias indices and the 95 % confidence intervals for $\kappa_{observed}$ were calculated to aid interpretation and comparison of $\kappa_{observed}$. Results of these calculations and the contingency tables from which they were made are shown in Appendix 18.

Figure 7.1. LAMP assay agreement with reference PCR results



LAMP RIME and LAMP *PfrA* were compared to the TBR PCR; LAMP *TgsGP* was compared to the *TgsGP* PCR; and LAMP *SRA*1 and SRALAMP_a were compared to the *SRA* PCR. Error bars show the 95 % confidence intervals for each of the kappa statistics.

7.4.1.2 LAMP test-retest agreement

Secondly, the repeatability of each LAMP assay was assessed. Two measures are considered. Firstly, the overall percentage agreement was calculated for each assay and secondly the agreement between any two repeats of an assay was calculated using Cohen's kappa statistic.

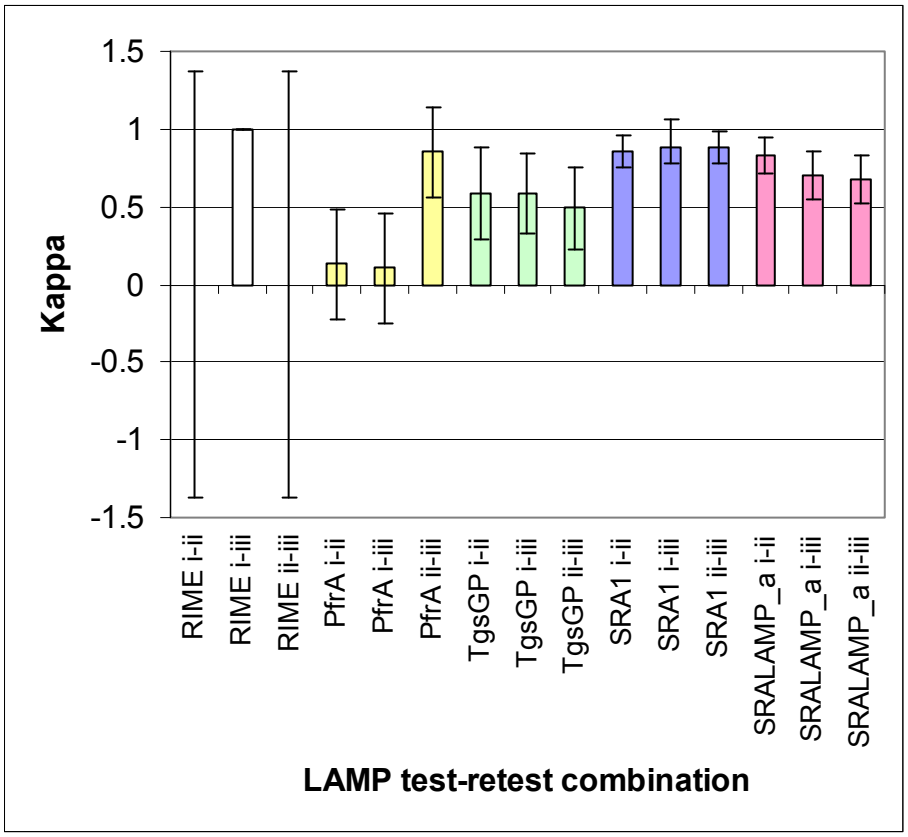
Table 7.1 shows the overall percentage agreement for each LAMP assay. For the *Trypanozoon* identification LAMP RIME was better than LAMP *PfrA*. For *T. b. rhodesiense*, LAMP *SRA*1 was better than SRALAMP_a.

Table 7.1. Overall percentage agreement for each LAMP assay applied in triplicate to 86 DNA samples

LAMP	Overall percentage agreement
RIME	97.67 %
PfrA	77.91 %
TgsGP	83.72 %
*SRA*1	89.53 %
SRALAMP_a	80.23 %

Figure 7.2 shows a summary of the agreement (quantified by Cohen's kappa statistic) for all test-retest combinations of each assay. The values of κ_{max}, the prevalence and bias indices and the 95 % confidence intervals for $\kappa_{observed}$ were also calculated to aid interpretation and comparison of $\kappa_{observed}$. The results of these calculations and the contingency tables from which they were made are shown in Appendix 19.

Figure 7.2. LAMP assay test-retest agreement



LAMP RIME i-ii indicates that the first repeat screen with the LAMP RIME assay was compared to the second repeat screen with the LAMP RIME assay, etc. Error bars show the 95 % confidence intervals for each of the kappa statistics.

7.4.1.3 Pentaplicate repeats for a subset of samples

Evaluation of LAMP assay reliability on control DNA samples was then extended by looking at the repeatability of the tests performed five times on a subset of samples. Table 7.2 shows the results when the LAMP assays were repeated five times alongside the reference PCR results (performed once to confirm the molecular identification of these samples). LAMP RIME detected all samples (all *Trypanozoon*s) with 100 % sensitivity, while LAMP *PfrA* gave two false negative results out of five repeats for one *T. b. gambiense* sample. LAMP *TgsGP* was

perfectly specific but failed to identify *T. b. gambiense* on four occasions. Finally, the LAMP assays for *SRA* were perfectly sensitive but both picked up false positives.

Table 7.2. Number of positive LAMP reactions out of five repeats

	PCR			Positive LAMP assays (n= 5)				
Sample	**TBR**	***SRA***	***TgsGP***	**RIME**	***PfrA***	***TgsGP***	**SRA LAMP_a**	***SRA*1**
1. Tira 24	+	-	-	5	5	0	1	1
2. Sikuda 28	+	-	-	5	5	0	0	1
6. Papol 33	+	-	-	5	5	0	1	0
7. Bumanda 146	+	-	-	5	5	0	0	0
8. Papol 264	+	-	-	5	5	0	0	0
5. Dal069 IM52	+	+	+	5	5	5	1	0
17. TH149	+	-	+	5	5	4	0	0
18. Bida 3CloneA	-	-	+	5	5	5	3	0
20. Katerema 311	+	-	+	5	3	3	0	0
21. Musikia CloneA	-	-	+	5	5	4	0	1
3. UGC	+	+	-	5	5	0	5	5
4. UGI	+	+	-	5	5	0	5	5
9. UGA 88	+	+	-	5	5	0	5	5
14. Papol 103	+	+	-	5	5	0	5	5
80. Rose Akinare	+	+	-	5	5	0	5	5

Samples highlighted in green are *T. b. brucei*, in yellow are *T. b. gambiense* and in pink are *T. b. rhodesiense*.

Table 7.3 (below) shows the overall percentage agreement of each LAMP assay with this subset of samples, calculated using the results tabulated above (see Table 7.2). Only LAMP RIME was perfectly reliable on these control DNA samples. However

since this subset did not contain non-*Trypanozoon*s, it was not possible to assess the reliability of LAMP RIME for generating true negative results.

Table 7.3. Overall percentage agreement for each LAMP assay in pentaplicate (15 DNA samples)

LAMP	Overall percentage agreement
RIME	100 %
PfrA	93.33 %
TgsGP	80 %
*SRA*1	80 %
SRALAMP_a	73.33 %

Fleiss' kappa [271] was used to quantify the agreement across the five replicates for the subspecies specific LAMP assays (LAMP *SRA*1, SRALAMP_a and LAMP *TgsGP*) (see Table 7.4). This gives a chance corrected measure of agreement. Accordingly, the novel assay for *T. b. rhodesiense* was most reliable, while the two novel assays were less reliable, but none show perfect agreement.

Table 7.4. Fleiss' kappa statistic for agreement across five repeats

LAMP Assay	SRALAMP_a	*SRA*1	*TgsGP*
Kappa	0.753	0.829	0.769

7.4.2 LAMP and PCR assay reproducibility when applied to field samples

Having considered the repeatability of LAMP on control DNA samples, reliability was then assessed using human patient and cattle blood samples stored on Whatman

FTA cards. Table 7.5 summarises the results seen when the TBR PCR, *SRA* PCR, LAMP RIME, LAMP *SRA*1 and SRALAMP_a assays were applied in triplicate to cattle and human patient blood samples.

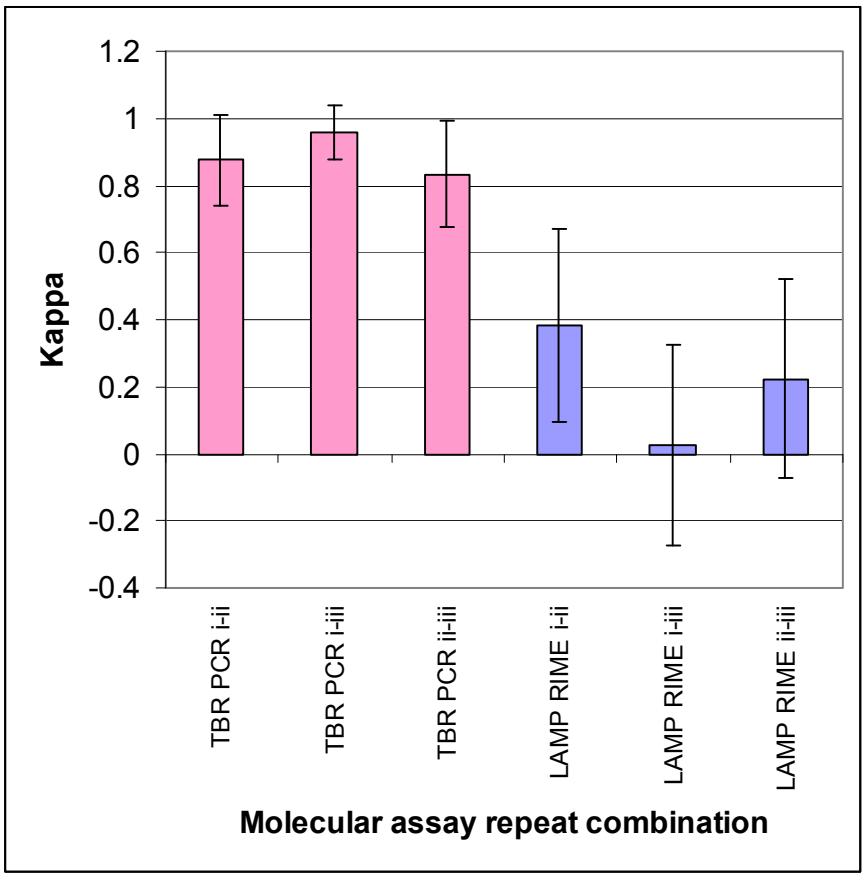
Table 7.5. Repeatability of PCR and LAMP assays applied to cattle blood samples

Cattle blood samples					
	TBR PCR	***SRA* PCR**	**LAMP RIME**	**LAMP *SRA*1**	**SRA LAMP_a**
All +	21	0	4	1	1
All -	23	0	18	32	44
All insufficient DNA[a]		36			
Total	48	48	48	48	48
% agreement	91.66	75.00	45.83	68.75	93.75
Human patient blood samples					
	TBR PCR	***SRA* PCR**	**LAMP RIME**	**LAMP *SRA*1**	***SRA* LAMP_a**
All +	52	27	41	34	3
All -	0	1	0	5	20
All insufficient DNA[a]		4			
Total	52	51	52	52	52
% agreement	100	62.74	78.85	75.00	44.23

[a]This category refers to the *SRA* PCR only

Figure 7.3 presents a graphical summary of the agreement for all test-retest combinations of the TBR PCR and LAMP RIME assays on the cattle samples. There were no *a priori* expectations about the outcome of these tests (i.e. the infection status of these samples was unknown). Agreement was quantified by Cohen's kappa statistic. The values of κ_{max}, the prevalence and bias indices and the 95 % confidence intervals for $\kappa_{observed}$ were also calculated to aid interpretation and comparison of $\kappa_{observed}$. The results of these calculations and the contingency tables from which they were made are shown in Appendix 20. The same analysis is not shown for the *SRA* PCR, LAMP *SRA*1 and SRALAMP_a since only two of the cattle samples were *SRA* PCR positive for at least one of the three repeats. Therefore the confidence intervals of any kappa estimates would be extremely wide.

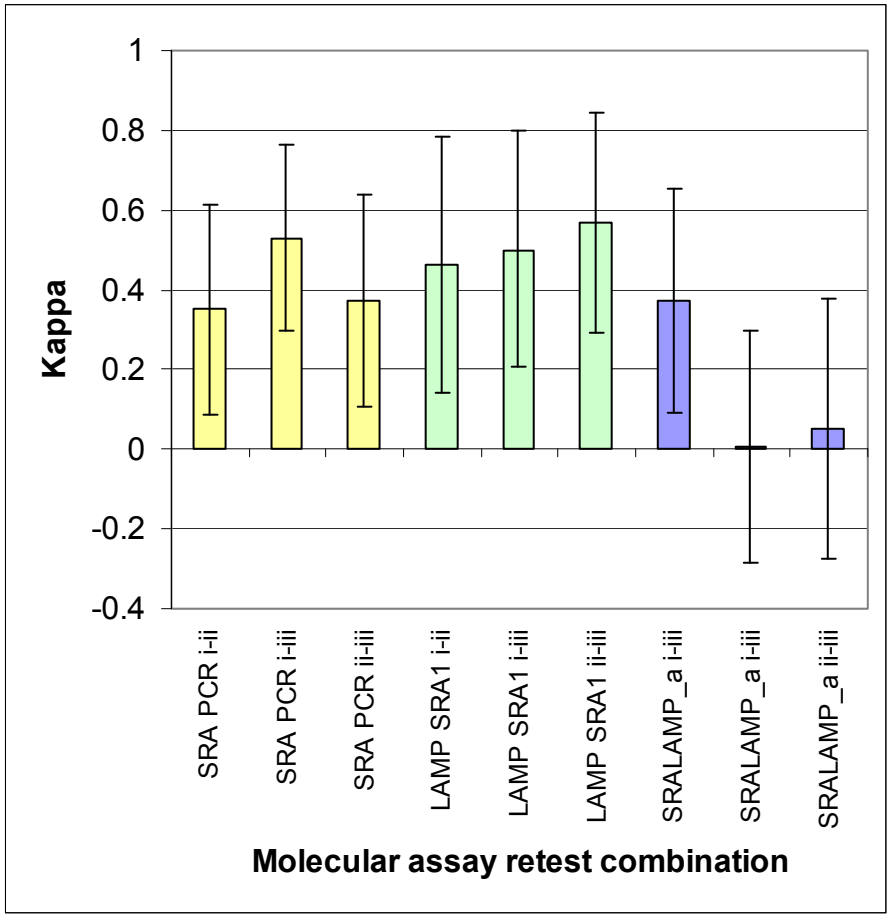
Figure 7.3. LAMP and PCR test-retest agreement using DNA prepared from cattle blood samples



TBR PCR i-ii indicates that the first repeat screen with the TBR PCR assay was compared to the second repeat screen with the TBR PCR assay, TBR PCR i-iii indicates that the first repeat screen with the TBR PCR assay was compared to the third repeat screen with the same assay and TBR PCR ii-iii indicates that the second repeat screen with the TBR PCR assay was compared to the third repeat screen with the same assay. Test-retest comparisons were also made for LAMP RIME repeats (first to second (i-ii), first to third (i-iii) and second to third (ii-iii)). Error bars show the 95 % confidence intervals for each of the kappa statistics.

Figure 7.4 presents a graphical summary of the agreement for all test-retest combinations of the *SRA* PCR, LAMP *SRA*1 and SRALAMP_1 assays when these were performed simultaneously on the human patient blood samples. All human samples were from parasite positive individuals. The TBR PCR identified all the samples as positive (see Table 7.5). None of these three molecular assays for the *SRA* gene generated consistent positive signals for all samples. Agreement was quantified by Cohen's kappa statistic. The values of κ_{max}, the prevalence and bias indices and the 95 % confidence intervals for $\kappa_{observed}$ were used to aid interpretation and comparison of $\kappa_{observed}$. The results of these calculations and the contingency tables from which they were are shown in Appendix 21.

Figure 7.4. LAMP and PCR test-retest agreement using DNA prepared from human blood samples



SRA PCR i-ii indicates that the first repeat screen with the *SRA* PCR assay was compared to the second repeat screen with the *SRA* PCR assay, *SRA* PCR i-iii indicates that the first repeat screen with the *SRA* PCR assay was compared to the third repeat screen with the same assay and *SRA* PCR ii-iii indicates that the second repeat screen with the *SRA* PCR assay was compared to the third repeat screen with the same assay. Test-retest comparisons were also made for LAMP *SRA*1 and SRALAMP_a repeats (first to second (i-ii), first to third (i-iii) and second to third (ii-iii)). Error bars show the 95 % confidence intervals for each of the kappa statistics.

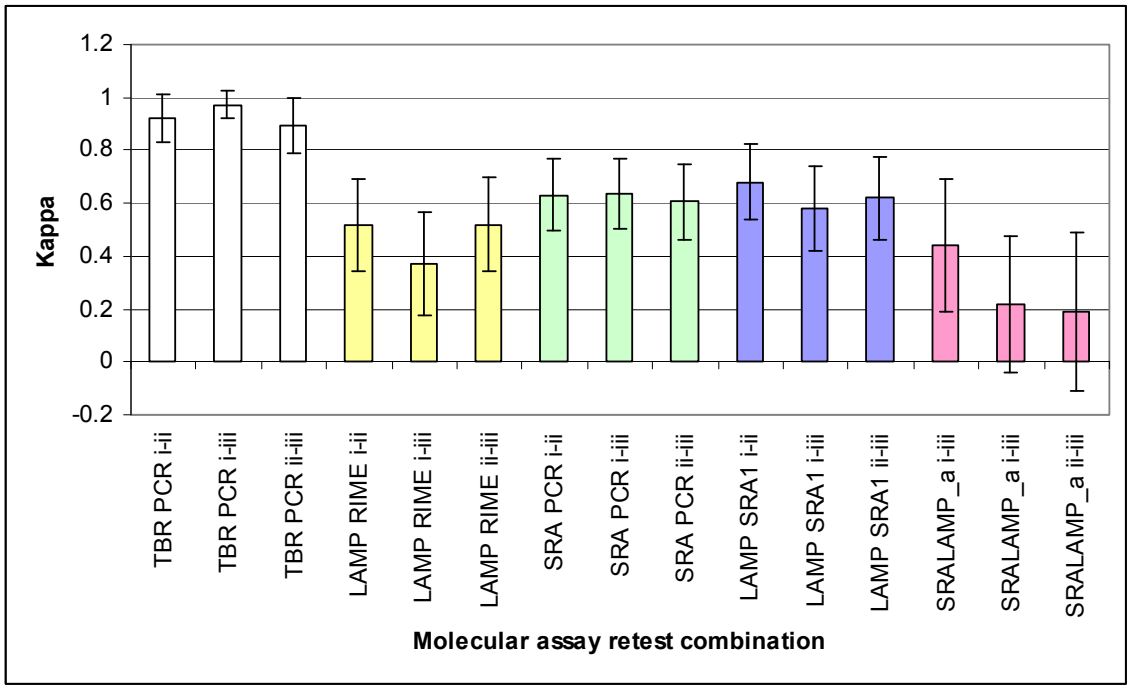
TBR PCR and LAMP RIME were also applied to these human samples. As mentioned above and in Table 7.5 all the TBR PCR were positive, whereas the

LAMP assay for *Trypanozoons* (LAMP RIME) showed only poor to fair test-retest agreement (κ = 0.071 to 0.305) with all 95 % confidence intervals including zero (see Appendix 21).

The data from the human and cattle blood samples was then pooled to increase the sample size, in order to make more confident statements about assay reliability. Using pooled data test-retest agreement was very good for the TBR PCR (κ = 0.893 to 0.973) and fair to moderate for LAMP RIME (κ = 0.370 to 0.518). *SRA* PCR showed good test-retest agreement (κ = 0.605 to 0.635). Similarly the published LAMP assay for *T. b. rhodesiense* (LAMP *SRA*1) showed moderate to good test-retest agreement (κ = 0.580 to 0.680), but SRALAMP_a showed only poor to moderate test-retest agreement (κ = 0.191 to 0.443).

Figure 7.5 presents a summary of the agreement for all test-retest combinations of the TBR and *SRA* PCR and the LAMP RIME, LAMP *SRA*1 and SRALAMP_a assays when all the data from cattle blood samples and human patient blood samples were pooled. Agreement was quantified by Cohen's kappa statistic. The values of κ_{max}, the prevalence and bias indices and the 95 % confidence intervals for $\kappa_{observed}$ were also calculated to aid interpretation and comparison of $\kappa_{observed}$. The results of these calculations and the contingency tables from which they were made are shown in Appendix 22.

Figure 7.5. LAMP and PCR test-retest agreement pooling all data from cattle and human samples



TBR PCR i-ii indicates that the first repeat screen with the TBR PCR assay was compared to the second repeat screen with the TBR PCR, TBR PCR i-iii indicates that the first repeat screen with the TBR PCR assay was compared to the third repeat screen with the same assay and TBR PCR ii-iii indicates that the second repeat screen with the TBR PCR assay was compared to the third repeat screen with the same assay. Test-retest comparisons were also made for LAMP RIME, *SRA* PCR, LAMP *SRA*1 and SRALAMP_a repeats (first to second (i-ii), first to third (i-iii) and second to third (ii-iii)). Error bars show the 95 % confidence intervals for each of the kappa statistics.

7.5 Discussion

This study was performed in order to explicitly evaluate the reliability of LAMP tests for the sleeping sickness parasites. Pan-*Trypanozoon* and sub-species specific *T. b. gambiense* and *T. b. rhodesiense* LAMP assays were considered. Firstly, two LAMP assays for *Trypanozoon*s (LAMP RIME and LAMP *PfrA*) and three subspecies specific assays (LAMP *TgsGP* for *T. b. gambiense* and LAMP *SRA*1 and

SRALAMP_a for *T. b. rhodesiense*) were applied in triplicate to 86 control DNA samples for test-retest analysis and comparison to the reference PCRs. This was extended to five repeats for a subset of the control DNA samples. Secondly, reliability was assessed using cattle and human blood samples that were representative of those that might be collected in the field as part of an epidemiological survey. They were screened in triplicate by the reference PCRs as well as LAMP RIME, LAMP *SRA*1 and SRALAMP_a. Unfortunately suitable samples for a similar reliability study of LAMP *TgsGP* were not available.

7.5.1 Reliability of the LAMP assays upon three repeated applications to the 86 control DNA samples

The published LAMP assay for *T. b. rhodesiense* (LAMP *SRA*1) consistently showed very good agreement ($\kappa > 0.8$), both with the reference PCR for the *SRA* gene, and in test-retest scenarios. Repeatability is apparent from the narrow range of kappa values which for test retest reliability varied by only 0.025, and for agreement to the reference PCR varied by only 0.046. Neither the prevalence nor bias indices give reason for concern that these kappa values might be artificially inflated or deflated and the confidence intervals were relatively narrow. However agreement was imperfect even when the marginal totals were accounted for; $\kappa_{observed}$ did not reach κ_{max}.

The agreement between each replicate of the novel LAMP assay for *T. b. rhodesiense* (SRALAMP_a) with the reference PCR was less consistent, with kappa varying over a range of 0.204. Kappa values for the test-retest reliability were also less strong and more variable than for LAMP *SRA*1. However even at its worst, test-retest reliability remained 'good' at $\kappa = 0.7$. Again, the confidence intervals were reasonably narrow, neither the prevalence nor bias indices give reason for concern that these kappa values might be artificially inflated or deflated and agreement was

imperfect, even when the marginal totals were accounted for; $\kappa_{observed}$ did not reach κ_{max}.

The published LAMP assay for *T. b. gambiense* (LAMP *TgsGP*) did not show consistent agreement with the reference PCR for the *TgsGP* gene. Although at the first run it showed very good agreement ($\kappa = 0.935$) with the PCR, at the second and third repeats this fell to $\kappa = 0.548$ and 0.554 respectively. Hence kappa varied by 0.387. Test-retest agreement was moderate in each instance. Notably, however the prevalence indices were high, both for agreement with the reference PCR and for test-retest calculations, reflecting the underlying asymmetry of the sample set, which contains a low proportion of *T. b. gambiense* samples. High prevalence indices can generate artificially low kappa values, and so this test might well be better than these statistics suggest. It would be useful to perform further validation studies on a larger sample set, or one that contains a higher proportion of *T. b. gambiense* samples. This would improve the precision of the kappa values, which at present have very wide 95% confidence intervals. Sample size guidelines are available in the literature [202]. They were not adhered to here with respect to *T. b. gambiense* since no more samples were available at the time of the study.

Similarly the distribution of *Trypanozoon* and non-*Trypanozoon* DNAs makes the sample set ill-suited for kappa based reliability measures of LAMP RIME and LAMP *PfrA*. Instead, we can consider the overall percentage agreement. Unlike kappa this does not measure true agreement, since it does not account for agreement that would occur by chance alone. However, it is useful here as a crude measure. Across three repeated applications to the 86 DNA samples perfect agreement was seen for LAMP RIME (all three results positive, or all three negative) for 84 samples, giving an overall percentage agreement of 97.67 %. LAMP *PfrA* was less repeatable; perfect agreement was seen for 67 samples giving an overall percentage agreement of 77.91 %. For the other three assays overall percentage agreement ranged from 80-90 %.

7.5.2 Reliability of the LAMP assays upon five repeated applications to a subset of 15 DNA samples

When reliability was investigated further by performing additional retest assays on a subset of samples LAMP RIME was more reliable than LAMP *PfrA* (according to the overall percentage agreement). Since both these assays are *Trypanozoon* specific, and no non-*Trypanozoon* samples were included in the subset, this reliability can only be interpreted in terms of the ability of the assay to reproducibly detect true positives.

For LAMP *SRA*1, SRALAMP_a and LAMP *TgsGP* agreement was described by overall percentage agreement and by Fleiss' kappa statistic, which is a measure of agreement beyond that expected by chance alone. None of these assays were perfectly reliable. Across the 5 repeats LAMP *SRA1* and SRALAMP_a were perfectly sensitive, but generated false positives, whereas LAMP *TgsGP* was perfectly specific but missed some true positives. However, the results were promising. Particularly, the pentiplicate subset repeat data supports an improved interpretation of LAMP *TgsGP* reliability compared to when the same assay was repeated in triplicate for all 86 samples. This reinforces the importance of having an appropriate distribution of samples within any sample set for any validity or reliability analyses. The full sample set was not ideal for a reliability study of a *T. b. gambiense* specific assay, since it did not contain very many *T. b. gambiense* samples. Unfortunately this was unavoidable within the limits of this study as there were no more *T. b. gambiense* samples available for use.

7.5.3 Reliability of the assays when applied to FTA card samples

The TBR PCR consistently showed very good agreement ($\kappa > 0.8$) when applied to cattle blood samples. Consistency is apparent from the relatively narrow range of kappa values which for test-retest reliability varied by only 0.125. When applied to human patient blood samples all the TBR PCR results for all the samples were

positive. Thus the results were in perfect agreement even though kappa cannot be calculated when the total observed positive-positive, or negative-negative agreements equals zero.

By contrast the LAMP assay for *Trypanozoons*, LAMP RIME, showed only poor to fair test-retest agreement both when it was applied to cattle blood samples and when it was applied to human patient blood samples. For the human samples the prevalence index was quite high (0.769 – 0.865), so it is possible that kappa is artificially deflated for agreement between LAMP RIME repeats on these samples. However, the prevalence index is less of a concern for the cattle samples, and kappa remains low. Furthermore, the observed kappa values fall far below their potential maximum values given the marginal totals.

When the data from the human and cattle samples was pooled test-retest agreement was very good (κ = 0.893 to 0.973) for the TBR PCR, but was fair to moderate (κ = 0.370 to 0.518) for LAMP RIME. By pooling the data the confidence intervals became less broad and do not include zero. Hence, agreement exceeds that expected by chance alone but LAMP RIME is less reliable than the TBR PCR.

The majority of the cattle blood samples did not contain sufficient DNA for the detection of a single copy gene, such as *SRA*, by PCR. Therefore reliability analyses were not specifically performed for the *SRA* molecular detection assays on this sample set. However, when applied to human patient blood samples the *SRA* PCR showed fair to moderate test-retest agreement. Although again the confidence intervals are broad, they do not include zero, so we can be confident that agreement exceeds that expected by chance alone. Having noted that the *SRA* PCR might be more reliable for human patient blood samples, than cattle blood samples, one can consider the reliability when these data sets were pooled. In this case the confidence

intervals were much narrower, and the kappa for test- retest agreement was consistently good.

When the LAMP *SRA*1 and SRALAMP_a assays for *T. b. rhodesiense* were applied to the DNA eluted from the human blood samples, LAMP *SRA*1 was more reliable. The prevalence indices were moderately high for both, so again, kappa might be artificially deflated. The confidence intervals did not include zero for LAMP *SRA*1, in contrast to SRALAMP_a. When the data were pooled LAMP *SRA*1 was the most reliable again.

Repeatability was worse on real sample material compared to the control DNA samples. For LAMP *SRA*1 test-retest reliability ranged from $\kappa = 0.857$ to $\kappa = 0.880$ on the control DNA samples. On the field derived FTA card samples this fell considerably and kappa ranged from 0.580 to 0.680. For SRALAMP_a a similar pattern was seen. With the control DNAs kappa ranged from 0.676 to 0.835, whereas with the field samples kappa ranged from 0.191 to 0.443. It is possible that the repeatability of these LAMP reactions on real samples might be improved if samples were collected, stored, and prepared for molecular analysis by another method. However, FTA cards provide an unrivalled sample collection and storage system. They contain a chemically treated fibre matrix that lyses cells, inactivates proteins and immobilises DNA, making them suitable for long term storage of blood samples without a cold chain or additional reagents. However, sparse parasite material may be unevenly distributed on the card, so that even for a positive sample taking just a few punches might not include parasite DNA [272]. In this study the fewer and smaller punches of FTA card material were used from the human samples compared to the cattle blood samples. The number and size of punches used with cattle samples has been optimized in a previous study (Heba Ahmed, PhD Thesis, University of Edinburgh). The protocol used with human samples has not been optimized, but given that parasite DNA is known to be much higher in *T. b.*

rhodesiense patients, and given the finite nature of these precious samples, it was deemed appropriate to use less material.

7.5.4 Reliability requirements for clinical diagnostic tests

Ideally, diagnostics would be perfectly reliable (as well as being perfectly accurate). However, this is not always true, and an imperfect test might still be the test of choice. Where diagnostics are imperfect, algorithms can be designed to reduce uncertainty. For example, in the UK, national standard methods exist which define the testing algorithms for HIV and *Chlamydia trachomatis* (VSOP 11 and VSOP 37, respectively. See http://www.hpa-standardmethods.org.uk/pdf_sops.asp#virology). Screening for HIV is by a fourth generation immunoassay which combines detection of the HIV p24 antigen and HIV antibody detection in the same test. When the specimen is unreactive, repeat screening depends upon the patient's history of likely exposure. When the sample is reactive screening is always repeated, first on the same sample, and then on a second sample. *Chlamydia* testing uses one of several commercially available nucleic acid amplification tests. Again, the testing algorithm requires confirmation of a positive result by repeat testing. The testing algorithm also defines the course of action in the face of intermediate or invalid results.

7.5.5 Conclusion

LAMP is not a perfectly reliable test and repeatability worsens when real samples are used. However, the reliability measures determined here cannot necessarily be extrapolated to samples processed differently. If and when LAMP is developed as a bedside diagnostic tool, reliability must not be taken for granted but should be quantified under that system. If might be necessary to perform more than one test per individual if LAMP does not prove to be sufficiently reliable. In terms of epidemiological surveillance and other research, perfect reliability might not be

necessary. However, the degree of unreliability must be quantified so that results are meaningful and comparable.

8 Chapter 8. Comparing simple methods for reading the results of LAMP reactions

8.1 Introduction

Loop-mediated isothermal amplification (LAMP) is promoted as a low technology alternative to traditional PCR that might be useful in resource poor settings [138]. To this end accurate and reliable interpretion of the LAMP is vital.

The LAMP reaction generates a mixture of stem loop DNAs of various stem lengths containing multiple loops of inverted repeats of the target sequence [123] (see Figure 1.2). These products can be detected in a variety of ways, the majority of which are not sequence-specific, as described in Chapter 1.

Visible turbidity was reported as a means to discern positive LAMP *PfrA* results [140], but not for the RIME [121] or *SRA* assays [122]. The LAMP *PfrA* assay contains 8 mM $MgSO_4$, whereas the RIME and *SRA* assays contain only 2 mM $MgSO_4$. The SRALAMP_a (Chapter 4) and LAMP *TgsGP* (Chapter 6) assays were designed to contain 8 mM $MgSO_4$ to facilitate turbidity based endpoint assessments.

Quant-iT PicoGreen [132] is a DNA intercalating dye that can be added to the LAMP reaction tube post-incubation. In the presence of LAMP amplified DNA the dye shows an orange to green colour change and fluoresces under UV light. While simple, addition of the dye is an extra processing requirement and further opening of reaction tubes may expose the laboratory to contamination by LAMP products; this contrasts with calcein, $MnCl_2$, and hydroxynaphthol blue that are added before incubation.

Calcein is a metal ion indicator, which responds to alterations in the chemical composition of the reaction mix as DNA amplification proceeds. Calcein and $MnCl_2$ are reported to produce an orange to green colour change, and to fluoresce under UV light in the presence of LAMP products [136]. Calcein and $MnCl_2$ are added alongside the other reagents before incubation. Initially, manganese ions combine with calcein to quench its fluorescence, and the reaction solution is orange. When the LAMP reaction proceeds in the presence of target DNA, the pyrophosphate ion by-product binds the manganese ions, releasing calcein. Calcein then binds to residual magnesium ions, the fluorescence increases and the reaction mix is green by eye.

Hydroxynaphthol blue is also a metal ion indicator which produces a violet to sky blue colour change in the presence of LAMP products [137]. It is added alongside the other reagents before incubation. The mechanism by which the colour change is generated is simpler than that described for calcein with $MnCl_2$ above. As the reaction progresses and pyrophosphate ion binds magnesium ions, the concentration of magnesium ions falls. Hydroxynaphthol blue acts as an indicator by directly responding to this change in magnesium ion concentration.

Here, turbidity, Quant-iT PicoGreen, hydroxynaphthol blue and calcein with $MnCl_2$ were evaluated for use with HAT specific LAMP reactions and were assessed using both well-characterised control DNA samples and human patient blood samples on Whatman FTA cards. Evaluation moved on to consider inter-observer reliability, which is particularly important when read out is subjective.

8.2 Aims

1. To establish the ease of use, sensitivity and cost of four simple endpoint detection methods with four HAT-specific LAMP assays

2. To compare turbidity (assessed by eye) with gel electrophoresis and UV illumination for LAMP *PfrA*, LAMP *TgsGP* and SRALAMP_a using 86 control DNAs
3. To determine the inter-rater reliability of two metal ion indicator methods for assessing LAMP endpoints.

8.3 Study outline

Firstly, purified trypanosome DNA was used to establish the ease of use and sensitivity of four simple endpoint detection methods (turbidity assessed by eye, colour change and fluorescence under ambient and UV light respectively with Quant-iT PicoGreen, colour change under ambient light with hydroxynapthol blue and colour change and fluorescence under ambient and UV light respectively with calcein and $MnCl_2$) using four LAMP assays (LAMP *PfrA*, LAMP RIME, LAMP *SRA*1 and SRALAMP_a). These were performed using a 10-fold dilution series of sample 86 whose concentration was measured using a NanoDrop (ThermoScientific) spectrophotometer.

For evaluating turbidity, each assay was performed once on the *T. b. rhodesiense* DNA dilution series. After turbidity assessment reaction products were subject to gel electrophoresis. LAMP *PfrA* and SRALAMP_a assays, were performed with Triton X-100 in the reaction buffer (minor modification to the published LAMP *PfrA* format). LAMP RIME and LAMP *SRA*1 were first performed according to their published protocols, then the concentration of dNTPs was increased from 0.2 to 1.4 mM and the concentration of $MgSO_4$ was increased from 2 to 8 mM in line with the reaction mix composition for LAMP *PfrA*.

For Quant-iT PicoGreen, LAMP *PfrA*, LAMP RIME, LAMP *SRA*1 and SRALAMP_a were all performed in duplicate on the *T. b. rhodesiense* dilution series described above with Triton X-100 in the reaction buffer (a minor modification to the published LAMP *PfrA* format). After the full LAMP reaction incubation time 5 µl of the LAMP product was aliquoted for gel electrophoresis. Then, 2 µl Quant-iT PicoGreen was added to one replicate and 5 µl Quant-iT PicoGreen was added to the second.

For hydroxynaphthol blue, LAMP *PfrA*, LAMP RIME, LAMP *SRA*1 and SRALAMP_a were performed once on the *T. b. rhodesiense* dilution series described above with Triton X-100 in the reaction buffer (a minor modification to the published LAMP *PfrA* format) with hydroxynaphthol blue. Products were also subject to gel electrophoresis.

For calcein with $MnCl_2$, LAMP *PfrA*, LAMP RIME, LAMP *SRA*1 and SRALAMP_a were all performed in triplicate on the *T. b. rhodesiense* dilution series described above with both reaction buffer compositions (Triton X-100 and Tween-20). Products were also assessed by gel electrophoresis

Secondly, 86 control DNA samples (Appendix 2) were used to validate the use of turbidity as a simple visual endpoint discrimination method for LAMP *PfrA*, SRALAMP_a and LAMP *TgsGP*. For LAMP *PfrA* the results were analysed for all 86 samples. For SRALAMP_a and LAMP *TgsGP* the statistical analysis excluded LAMP *PfrA* negative samples 56, 62 and 72, in line with other analyses made for these assays on this sample set.

Thirdly, a multi-observer study was performed in order to investigate the inter-observer reliability of the two metal ion indicator methods – colour change with hydroxynaphthol blue and colour change with calcein and $MnCl_2$. These methods

were chosen for their advantages as low cost, closed system methods. The study used sixty blood samples from human sleeping sickness patients spotted on to Whatman FTA cards. The SRALAMP_a assay was performed, in duplicate, for each sample, firstly including reaction included hydroxynaphthol and secondly with calcein and $MnCl_2$ using Tween-20 instead of Triton X-100. Products were subject to gel electrophoresis. The results from each observer were compared to the status of each sample, as defined by gel electrophoresis and UV illumination. The agreement between each observer and the gel result was quantified using Cohen's kappa statistic (κ) [157]. The overall level of agreement between all observers was then quantified [271] for both colour change detection methods.

8.4 Results

8.4.1 Ease of use and sensitivity of four simple endpoint detection methods

Four simple endpoint detection methods were assessed using four LAMP assays: LAMP *PfrA*, LAMP RIME, LAMP *SRA*1 and SRALAMP_a.

8.4.1.1 Turbidity

Positive LAMP *PfrA* reactions were detectable by visible turbidity up to and including a 1 x 10^{-4} dilution of the *T. b. rhodesiense* DNA. This was equal to the detection limit seen when the same reaction products were visualised by UV illumination after gel electrophoresis. Similarly, positive SRALAMP_a reactions were detectable by visible turbidity up to and including a 1 x 10^{-3} dilution of the *T. b. rhodesiense* DNA. This was equal to the detection limit seen when the same reaction products were visualised by UV illumination after gel electrophoresis.

Visible turbidity detection is not reported to be possible for LAMP RIME or *SRA*1. However, when the dNTP and $MgSO_4$ concentrations were simultaneously increased

turbidity could be observed up to and including a 1 x 10^{-4} dilution of the *T. b. rhodesiense* DNA for both assays. This was equal to the detection limit seen when the same reaction products were visualised by UV illumination after gel electrophoresis. Therefore, turbidity was as sensitive as gel electrophoresis for detecting LAMP positive end-points for all four assays and no false positives were observed. These results are shown alongside the detection limits with other endpoint readout formats (see Table 8.1).

8.4.1.2 Quant-iT PicoGreen

Addition of 2 µl or 5 µl Quant-iT PicoGreen allowed positive reactions to be discerned under ambient and UV light for all four LAMP assays. Quant-iT PicoGreen was not tested for LAMP *TgsGP*. The detection limit for each assay with these formats is shown in Table 8.1. For LAMP RIME, LAMP *SRA*1 and SRALAMP_a colour and fluorescence showed perfect agreement with gel electrophoresis regardless of the volume of Quant-iT PicoGreen. For LAMP *PfrA* with 2 µl Quant-iT PicoGreen, colour and fluorescence based detection disagreed with the results by gel electrophoresis in two instances. Gel positive, but colour change and fluorescence negative endpoints were seen with the 1 x 10^{-4} dilution and gel negative but colour change and fluorescence positive endpoints were seen with the 1 x 10^{-5} dilution. However, no disagreements were seen when 5 µl Quant-iT Picogreen were used. An example of the colour change seen with Quant-iT PicoGreen can be seen in Figure 8.1.

8.4.1.3 Hydroxynaphthol blue

Hydroxynaphthol blue also allowed positive reactions to be discerned under ambient light for all four LAMP assays (Table 8.1). Hydroxynaphthol blue induced colour change showed perfect agreement with detection by gel for all assays except SRALAMP_a when one gel positive assay (corresponding to the 1 x 10^{-4} dilution) appeared violet (negative). No false positives were seen with hydroxynaphthol blue

versus the gel. For the LAMP *PfrA*, RIME and SRALAMP_a assays, inclusion of hydroxynaphthol blue had no effect on the detection limit. However the sensitivity of the LAMP *SRA1* was reduced when hydroxynapthol blue was added compared to those results seen with turbidity or Quant-iT PicoGreen (see Table 8.1). An example of the colour change seen with hydroxynaphthol blue can be seen in Figure 8.1.

8.4.1.4 Calcein and $MnCl_2$

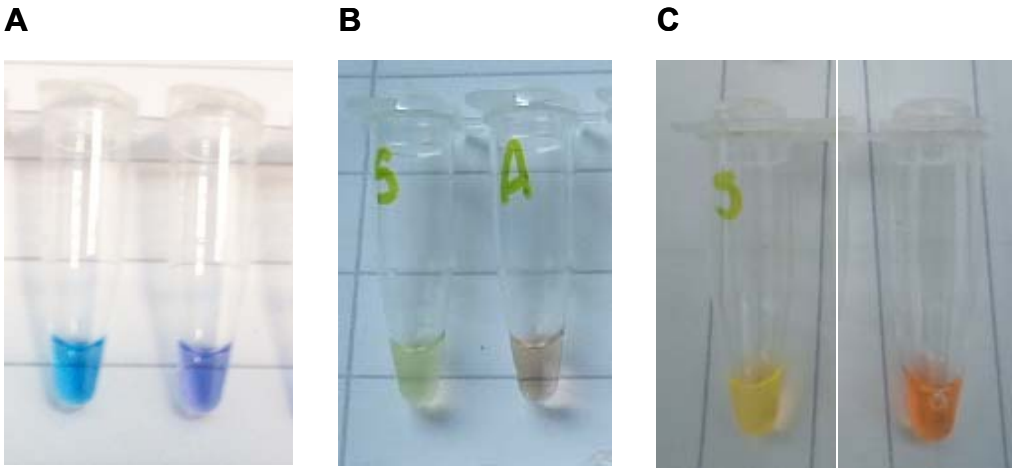
The calcein and $MnCl_2$ method was found to give results of variable quality. An example of the colour change seen with calcein and $MnCl_2$ can be seen in Figure 8.1. No positive LAMP amplification was seen with the RIME assay, with either reaction buffer, despite running each assay in triplicate for the full dilution series. Neither were any LAMP positive endpoints seen for the LAMP *PfrA* or LAMP *SRA1* reactions with 0.1% Triton X-100 containing LAMP buffer, despite running each assay in triplicate for the full dilution series. However, amplification was seen when 0.1% Tween-20 was used with the LAMP *PfrA*, SRALAMP_a and LAMP *SRA*1 reactions. The detection sensitivity for each of these assays varied over the three repeats and colour changes and fluorescence were sometimes ambiguous and did not always agree with the result determined by gel electrophoresis. SRALAMP_a amplification was seen for both reaction buffer compositions. Therefore this was the only reaction for which LAMP amplicons were detected when Triton X-100 was included with calcein and $MnCl_2$. Again the detection limit was inconsistent and did not always agree with the colour change and fluorescence observations (see Appendix 23).

Furthermore, the inclusion of calcein and $MnCl_2$ seemed to reduce the absolute sensitivity of the assay as compared to results seen with turbidity or Quant-iT Picogreen, where extra reagents were not added to the reaction mix.

Table 8.1. Sensitivity with different assays and detection methods with a 10 fold dilution series of 13.6 nM *T. b. rhodesiense* DNA.

		PfrA	**RIME**	***SRA1***	**SRALAMP_a**
Turbidity	Turbidity	1×10^{-4}	1×10^{-4}	1×10^{-4}	1×10^{-3}
	Gel	1×10^{-4}	1×10^{-4}	1×10^{-4}	1×10^{-3}
Quant-iT PicoGreen (2 µl)	Colour	1×10^{-3}	1×10^{-5}	1×10^{-4}	1×10^{-3}
	Fluorescence	1×10^{-3}	1×10^{-5}	1×10^{-4}	1×10^{-3}
	Gel	1×10^{-4}	1×10^{-5}	1×10^{-4}	1×10^{-3}
Quant-iT PicoGreen (5 µl)	Colour	1×10^{-4}	1×10^{-5}	1×10^{-4}	1×10^{-4}
	Fluorescence	1×10^{-4}	1×10^{-5}	1×10^{-4}	1×10^{-4}
	Gel	1×10^{-4}	1×10^{-5}	1×10^{-4}	1×10^{-4}
Hydroxynaphthol blue	Colour	1×10^{-5}	1×10^{-5}	1×10^{-3}	1×10^{-3}
	Gel	1×10^{-5}	1×10^{-5}	1×10^{-3}	1×10^{-4}

Figure 8.1. Colour change reactions with hydroxynaphthol blue, calcein with $MnCl_2$ and Quant-iT Picogreen.



Panel A shows the colour change seen with hydroxynapthol blue, panel B shows the colour change seen with calcein and MnCl2 and panel C shows the colour change seen with Quant-iT PicoGreen.

8.4.2 Turbidity for LAMP *PfrA*, SRALAMP_a and LAMP *TgsGP*

Turbidity was compared to detection by gel electrophoresis for three repeat screens of the total sample set for LAMP *PfrA*, SRALAMP_a and LAMP *TgsGP*. The results of these comparisons are given in two by two tables in Appendix 24, from which summary statistics were calculated (see Tables 8.2, 8.3 and 8.4 and Figure 8.2).

For LAMP *PfrA* kappa ranged from 0.554 to 0.765. The confidence intervals around these estimates are wide, reflecting the scarcity of LAMP *PfrA* negative samples in the set. Despite this none of the confidence intervals span zero, supporting the hypothesis that agreement between turbidity and gel electrophoresis is significantly better than would be expected by chance. For SRALAMP_a kappa ranged from 0.475 to 0.951 and for LAMP *TgsGP* from 0.751 to 0.868. The confidence intervals are generally slightly narrower for LAMP *TgsGP* kappa, and most narrow for SRALAMP_a kappa, reflecting the distribution of *T. b. gambiense* and *T. b.*

rhodesiense samples in the set. Again these confidence intervals do not span zero. In all but one instance (the second repeat screen with LAMP *PfrA*) turbidity detects gel positive LAMP endpoints with 100 % specificity. Sensitivity is variable both within and between assays.

Table 8.2. Turbidity by eye compared to UV visualisation of LAMP products after gel electrophoresis for LAMP *PfrA*, SRALAMP_a and LAMP *TgsGP*

	Repeat 1	**Repeat 2**	**Repeat 3**
Kappa (95 % CI)	0.765 (0.610 – 0.920)	0.554 (0.057 – 1.050)	0.589 (0.239 – 0.989)
Sensitivity (95 % CI)	88.06 % (77.91 – 94.08 %)	97.59 % (91.12 – 99.85 %)	93.90 % (86.18 – 97.70 %)
Specificity (95 % CI)	100 % (80.21 – 100 %)	66.67 % (20.24 – 94.37 %)	100 % (45.41 – 100 %)
NPV (95 % CI)	70.37 % (51.35 – 84.32 %)	50.00 % (15.00 – 85.00 %)	44.44 % (18.84 – 73.37 %)
PPV (95 % CI)	100 % (92.69 – 100 %)	98.78 % (92.76 – 99.99 %)	100 % (94.30 – 100 %)

Table 8.3. Turbidity by eye compared to UV visualisation of LAMP products after gel electrophoresis for SRALAMP_a

	Repeat 1	Repeat 2	Repeat 3
Kappa (95 % CI)	0.475 (0.275 – 0.676)	0.951 (0.885 – 1.018)	0.928 (0.847 – 1.008)
Sensitivity (95 % CI)	44.44 % (29.53 – 60.43 %)	94.87 % (82.21 – 99.48 %)	93.33 % (84.02 – 97.73 %)
Specificity (95 % CI)	100 % (90.98 – 100 %)	100 % (90.42 – 100 %)	100 % (89.07 – 100 %)
NPV (95 % CI)	70.15 % (58.29 – 79.82 %)	95.65 % (84.66 – 99.61 %)	92.68 % (79.88 – 98.17 %)
PPV (95 % CI)	100 % (77.31 – 100 %)	100 % (88.80 – 100 %)	100 % (90.01 – 100 %)

Table 8.4. Turbidity by eye compared to UV visualisation of LAMP products after gel electrophoresis for LAMP *TgsGP*

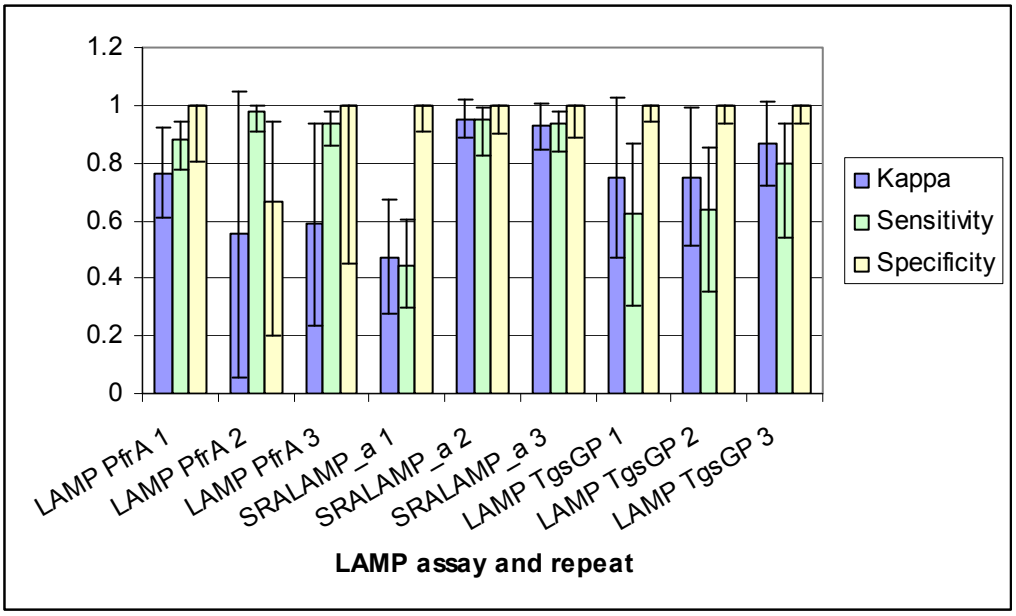
	Repeat 1	Repeat 2	Repeat 3
Kappa	0.751	0.752	0.868
(95 % CI)	(0.474 – 1.028)	(0.515 – 0.989)	(0.721 – 1.015)
Sensitivity	62.50 %	63.64 %	80.00 %
(95 % CI)	(30.38 – 86.51 %)	(35.19 – 85.02 %)	(54.05 – 93.72 %)
Specificity	100 %	100 %	100 %
(95 % CI)	(94.16 – 100 %)	(93.93 – 100 %)	(93.60 – 100 %)
NPV	96.15 %	94.74 %	95.77 %
(95 % CI)	(88.84 – 99.14 %)	(86.84 – 98.33 %)	(87.81 – 99.04 %)
PPV	100 %	100 %	100 %
(95 % CI)	(51.09 – 100 %)	(59.56 – 100 %)	(71.8 – 100 %)

To aid interpretation of kappa the prevalence and bias indices and the maximum attributable kappa were also calculated (Table 8.5).

Table 8.5. Prevalence and bias indices, and the maximum attributable kappa for turbidity versus gel electrophoresis with LAMP *PfrA*, SRALAMP_a and LAMP *TgsGP*

	LAMP *PfrA* repeat screen			SRALAMP_a repeat screen			LAMP *TgsGP* repeat screen		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
κ_{max}	0.765	0.851	0.589	0.475	0.951	0.928	0.751	0.752	0.868
Prevalence index	0.465	0.919	0.849	0.373	0.084	0.048	0.843	0.819	0.675
Bias index	0.093	0.012	0.058	0.241	0.024	0.036	0.036	0.048	0.036

Figure 8.2. Kappa, sensitivity and specificity, with 95 % confidence intervals for the agreement between turbidity and endpoint detection by gel electrophoresis for three LAMP assays



8.4.3 Multi-observer study

Inter-reader reliability of the hydroxynaphthol blue and calcein with $MnCl_2$ methods were evaluated using a multi-observer study with the SRALAMP_a assay applied to human patient blood samples stored on Whatman FTA cards.

8.4.3.1 Participants

Thirty three volunteers participated in this study. There was a strong occupational bias towards the biomedical sciences. Eighteen participants self-defined as biological scientists, six were veterinarians (of which, three also described themselves as biologists) and one was a medical doctor. Nine participants did not place themselves into any of the above categories. Nine participants reported some previous experience with diagnostic tests, which require a colour or colour change to be observed, although in most cases this experience was limited. Twenty one of the participants were female, twelve were male. Age was not surveyed.

8.4.3.2 Questionnaire

All observers said that they found the violet to blue colour change, seen with hydroxynaphthol blue, easier to use than the orange to green colour change seen with calcein and $MnCl_2$.

When asked, 'In your opinion, how easy is it to see the violet to sky blue /orange to green colour difference?' 73 % of observers found the colour change with hydroxynaphthol blue quite easy to see, whereas 94 % of the observers found the colour change with calcein and $MnCl_2$ very difficult to see.

When asked 'In your opinion, using this colour change, how many of the samples were easy to rate as positive or negative?' all participants found most or some of the samples easy to score with hydroxynaphthol blue.

All participants found very few or no samples easy to score with calcein and $MnCl_2$.

Of those who had some previous experience interpreting diagnostic tests both methods were generally considered to be less easy to interpret than their previous experiences.

Eight observers commented on a difference in turbidity between the positive and negative controls for the calcein and $MnCl_2$ method. Two participants found it easier to score the samples using this turbidity difference than with the colour difference seen with hydroxynaphthol blue. The participants were not closely questioned as to how they made their decision; it is possible that several more made their judgements on a similar basis.

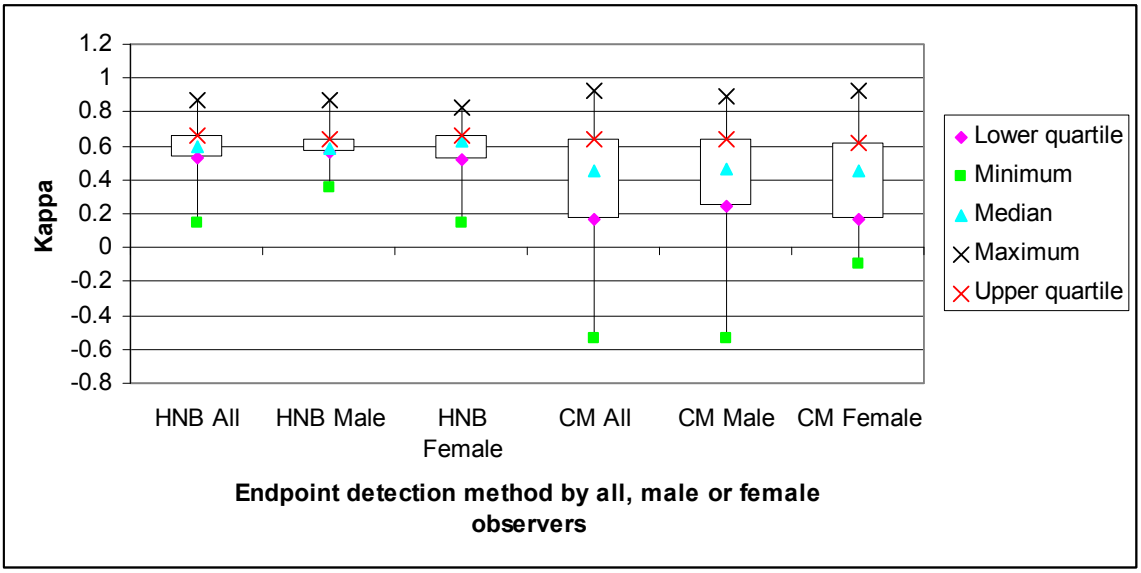
8.4.3.3 Individual observer agreement with reference standard method

Cohen's kappa statistic (κ) was used to compare the agreement of observers with the results according to gel electrophoresis. For hydroxynaphthol blue agreement ranged from $\kappa = 0.145$ (95 % CI -0.139 – 0.429) to $\kappa = 0.870$ (95 % CI 0.726 – 1.013). For calcein and $MnCl_2$ agreement ranged from $\kappa = -0.533$ (95 % CI -0.747 – -0.319) to $\kappa = 0.930$ (95 % CI 0.834 – 1.026). The range of kappa for the agreement between gel and colour change is summarised in Figure 8.3 below. This agreement is much more variable for calcein with $MnCl_2$ than for hydroxynaphthol blue, as seen by the total range, and more importantly the interquartile range.

In 66.67 % of observers the agreement between the hydroxynaphthol blue colour change and the results seen by gel electrophoresis were better than agreement between calcein $MnCl_2$ colour change and gel (n = 22/33; 95 % CI 49.52 – 80.34 %).

Since colour blindness is more prevalent among males the data were analysed for gender specific differences. The range of kappa for the agreement between gel and colour change is summarised in Figure 8.3 below. As well as showing the differences between hydroxynaphthol blue and calcein with $MnCl_2$, this box plot also breaks down the data by sex. For hydroxynaphthol blue the spread seems slightly increased for females, both in terms of the interquartile and absolute range. For calcein with $MnCl_2$ the absolute range has a greater spread for males, but the interquartile range is slightly wider for females. There are no large differences between the sexes for either method.

Figure 8.3 Agreement between the endpoint assessed by colour change (hydroxynapthol blue or calcein-$MnCl_2$) and gel electrophoresis for thirty three observers



HNB is hydroxynapthol blue, CM is calcein with $MnCl_2$.

8.4.3.4 Inter-observer agreement

The method of Fleiss, Levin *et al.* [271] was used to quantify the agreement of all observers for each method (Fleiss' kappa). For hydroxynaphthol blue, the Fleiss' kappa value of 0.693 was significantly different from zero - or no agreement, ($P<0.0001$). For calcein and $MnCl_2$ the Fleiss' kappa value of 0.209 was seen. This was also significantly different from zero ($P<0.0001$). Agreement between many observers was much higher for the hydroxynaphthol blue method.

8.4.4 Reagent costs

Quant-iT PicoGreen was the most expensive of the methods used. When 5µl of this reagent is added per reaction the cost is £116.00 for 100 reactions. By sharp contrast, the costs of turbidity, hydroxynaphthol blue and calcein with $MnCl_2$ are all less than £0.001 for 100 reactions (see Tables 8.6 and 8.7).

Table 8.6 Reagent costs and suppliers at the time of the study (2009)

Reagent	Supplier	Catalogue number	Cost
Quant-iT PicoGreen	Invitrogen, UK	P7581	£252 for 1 ml
Hydroxynaphthol blue	Sigma Aldrich, UK	33936	£33.70 for 10 g
Calcein	Sigma Aldrich, UK	C0875	£77.30 for 5 g
$MnCl_2$	Sigma Aldrich, UK	M8054	£25.80 for 100 g

Table 8.7. Reagent costs per 100 reactions (based on the costs described in Table 8.6 above)

Method of endpoint detection	Cost per 100 reactions
Quant-iT PicoGreen	£116.00
Turbidity	£0.00
Hydroxynaphthol blue	£0.0006
Calcein and $MnCl_2$	£0.00073

8.5 Discussion

LAMP is promoted as a low technology diagnostic tool for resource poor settings [138] and simple visual discrimination of the test result is perceived as an important factor for recommending the method as a straightforward diagnostic [137].

However, LAMP results can be read in a variety of ways. Complex sequence specific [131] and high technology, real-time turbidimetry [130] approaches are useful during assay design and optimisation. LAMP products may also be visualised directly following gel electrophoresis by UV trans-illumination. More simply, turbidity can be generated as a by-product of DNA amplification [124]. Several colour change methods for reading the result within the reaction tube have also been developed, including the use of DNA intercalating dyes: Quant-iT PicoGreen [132], SYBR green [133, 134] and propidium iodide [134] and the metal ion indicator methods: calcein alone [135], calcein with $MnCl_2$ [136] and, most recently, hydroxynaphthol blue [137]. The metal ion indicators have provided the simplest approach to date; they are added alongside the other reagents, before incubation, so that amplification and detection are combined in single processing step, within a closed tube system. The colour changes can be visualised by eye, without special lighting, and they are inexpensive.

In the present work Quant-iT PicoGreen was the most expensive of the methods used. When 5 µl of this reagent is added per reaction the cost is £116.00 for 100 reactions. In contrast, the costs of turbidity, hydroxynaphthol blue and calcein with $MnCl_2$ are all less than £0.001 for 100 reactions.

None of the methods assessed here confirm that LAMP amplification corresponds to its intended target. It is assumed that all LAMP amplicons arise from the target

region. As stated by Mori *et al.* [130] '*The LAMP method uses four primers that recognize six regions on the target nucleic acid, so that specificity is extremely high. For this reason, in the LAMP method, detection can be done without checking the amplification product during or after the amplification*'. Melt curve analysis supports this hypothesis, as demonstrated, for example, during the development of the LAMP RIME and *SRA*1 assays [121, 122]. However the possibility of mis-priming remains. By contrast, mis-priming during PCR can be easily detected since it will almost always give rise to a different size product which can easily be distinguished by gel electrophoresis.

Turbidity enabled sensitive and specific endpoint discrimination for LAMP *PfrA* and SRALAMP_a but is not visible for LAMP RIME or LAMP *SRA*1 using published protocols. However, by adjusting the concentration of dNTPs and $MgSO_4$, turbidity could also be visualised for both LAMP RIME and LAMP *SRA*1, though the effect of these changes on the sensitivity and specificity of these assays has not been quantified and further validation work would be required before these changes could be applied. Since other simple methods, principally the hydroxynaphthol blue format, can be used with both these assays, more extensive turbidity validation for LAMP RIME and LAMP *SRA*1 was not performed.

Quant-iT PicoGreen enabled easy visual endpoint discrimination for all assays; the colour and fluorescence were more easily assessed when 5 µl (rather than 2 µl) of this reagent but the extra cost is significant for this expensive assay. Simple, sensitive and specific endpoint discrimination was also possible for all assays using the hydroxynaphthol blue method. For LAMP RIME, *PfrA* and SRALAMP_a inclusion of hydroxynapthol blue did not reduce the assay sensitivity, confirming previous work [137] that this reagent does not inhibit the LAMP reaction. However, a ten fold reduction in the detection limit was observed for the LAMP *SRA*1 assay with hydroxynaphthol blue compared to LAMP *SRA*1 without any additional reagents in the reaction mix. Goto *et al.* [137] also reported Mn^{2+} inhibition of

LAMP, which may explain the total inhibition of LAMP RIME, and partial inhibition of LAMP *PfrA*, *SRA1* and SRALAMP_a assays found in the present work when calcein and Mn^{2+} was added.

When turbidity was compared to gel electrophoresis for LAMP *PfrA*, SRALAMP_a and LAMP *TgsGP* sensitivity, specificity, negative and positive predictive values and kappa were all variable across the three repeat screenings (this compromising variation was observed by a single reader, but it is not clear whether this is intra-reader variability or intrinsic variability of turbidity). Generally, turbidity was seen specifically for gel positive endpoints for all three assays, but the sensitivity of turbidity was lower and more variable. It should be noted that this might be more a reflection of the individual observer than the characteristics of the turbidity detection format. Since visual turbidity is a subjective assessment each observer will make his or her own threshold at which they define a reaction tube as cloudy enough to be counted as positive. A high threshold will predispose the reader towards recognizing positive reactions specifically but with low sensitivity. It should be noted that the confidence intervals around kappa and the accuracy estimate vary for each assay (they are much wider for LAMP *PfrA*) owing to the composition of the sample set, in which the proportions of LAMP positive and negative results are not equal for the three assays.

This work shows that each detection method should be validated for any given LAMP assay. This is particularly important for the turbidity and metal ion indicator approaches, which detect changes in the chemical composition of the reaction mix rather than amplified DNA. From initial work hydroxynaphthol blue was found to be the better of the two metal ion indicator methods tested, while the calcein and $MnCl_2$ method reduced LAMP reaction sensitivity, and was sensitive to the LAMP reaction buffer composition.

Nevertheless both hydroxynapthol blue and calcein with $MnCl_2$ were taken forward for assessment of inter-reader reliability. These methods were chosen for their advantages as closed system methods and their low cost. By contrast Quant-iT PicoGreen is expensive, and breaks the closed system. Turbidity was excluded because it is not possible for all published LAMP assays for HAT.

Inter-reader variability is an issue that has been largely ignored in the LAMP literature to date, and should be addressed where subjective end points are involved. In previous work end point interpretations have been made by one observer and to our knowledge no large scale, multi-observer studies have been performed with any of the LAMP endpoint detection methods. As Sadatsafavi *et al.* have emphasised, '*any attempt to generalize the performance of a subjective diagnostic method should take into account the sample variation in both cases and readers'* [273]; they further highlight the need for a large group of observers to be used.

The agreement across all observers was better for hydroxynaphthol blue tests (κ = 0.693) than for calcein with $MnCl_2$ (κ = 0.209), and, for 66 % of observers the hydroxynaphthol blue to gel agreement was better than the calcein-$MnCl_2$ to gel agreement. All observers said they found the hydroxynaphthol blue colour change easier to see. Several observers commented on a turbidity difference between positives and negatives for the calcein-$MnCl_2$ method. Six of the 11 observers whose calcein-$MnCl_2$ to gel agreement was better than that for hydroxynaphthol blue commented on this turbidity difference. The orange to green colour change may be less reliable than this report suggests.

The present work has shown that hydroxynaphthol blue is the better of the two metal ion indicator methods tested. Not only is it easier to see, but it also gives better inter-reader reliability and more consistent agreement with the presence of the DNA amplicon, assessed by gel. However, Cohen's kappa statistic for the agreement

between colour change (assessed by an individual observer) and the presence of DNA amplicon (assessed by gel) ranged from $\kappa = 0.145$ to $\kappa = 0.870$. Therefore, hydroxynaphthol blue could be well suited as a tool to increase the efficiency of large scale screening and monitoring efforts but, when more than one individual is involved in LAMP screening, training and quality control will be necessary to reduce inter-observer variation. This process might be aided by generating a colour swatch card against which assay results can be compared. A quantitative colour measure, using some kind of spectrophotometric device would remove this variability, although this would need to be suitable for a low-resource, African setting.

In conclusion, hydroxynaphthol blue was found to be the best method for easy, inexpensive, accurate and reliable interpretation of LAMP assays for human African trypanosomiasis. The violet to sky blue colour change was easy to see and was consistently interpreted by independent observers. A range of problems was found with the other methods. Visible turbidity is not possible for all LAMP HAT assays. Quant-iT PicoGreen performed excellently, but opening the reaction tube exposes the laboratory to product contamination. It is also significantly more expensive than the other methods. With calcein and $MnCl_2$ the four assays showed a range of partial to total inhibition and the colour change was difficult to see, leading to poor agreement between several independent observers.

However, hydroxynaphthol blue is not perfect. Here, the agreement between amplicon detection by gel electrophoresis and colour change with hydroxynaphthol blue is variable and imperfect for different observers. Therefore while hydroxynaphthol blue is a promising method for rapid, low-technology LAMP endpoint detection, further work is required to develop methods that will assist different observers to make consistent interpretations of the same colour change.

9 Chapter 9. Discussion

9.1 Background

Accurate diagnostics are required to guide treatment decisions for sleeping sickness (human African trypanosomiasis – HAT) because symptoms are non-specific and the available drugs are either too toxic or too complex to administer to unconfirmed cases. Diagnostics are needed to direct both who and how to treat. *T. b. rhodesiense* and *T. b. gambiense* must be differentiated, as must the early and late stages of disease, since they demand different treatment protocols. Aside from case detection and management, accurate diagnostics are needed for disease surveillance, monitoring of public health interventions and epidemiological studies. Tools that enable surveillance, intervention monitoring and epidemiological studies of the zoonotic reservoir of *T. b. rhodesiense* are also urgently required to coordinate integrated disease control activities. The currently available diagnostics, and their shortcomings are fully described in Chapter 1. Suffice to say that improved diagnostics, which are sensitive, specific and applicable in poorly resourced, peripheral health care facilities, would be extremely useful in the continued fight against sleeping sickness.

9.1.1 The development of LAMP diagnostics for sleeping sickness: a potted history

In 2000 a novel nucleic acid amplification method, LAMP (loop-mediated-isothermal amplification) was published [123] and LAMP was soon being promoted as a potential point of care diagnostic in the developing world:

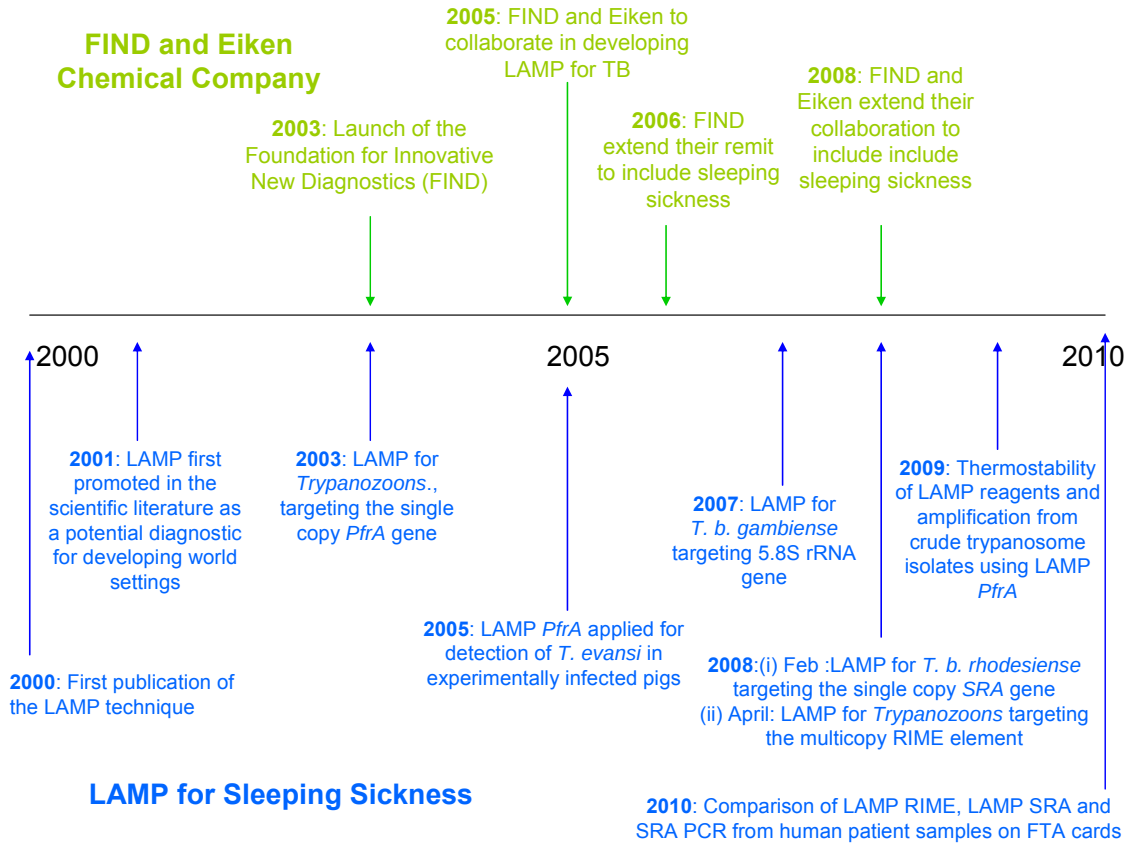
'*By the use of turbidity measurement of the LAMP method a small, simple, economically feasible and safe DNA detection device could be established. The*

development of such a device could realize the genetic point-of-care (gPOC) testing at the bedside or in underdeveloped nations in the near future.'[124][1]

In 2003 LAMP was first applied to human African trypanosomes in an assay designed to detect all members of the subgenus *Trypanozoon* [140]. Four months previously, the Foundation for Innovative New Diagnostics (FIND) had been launched. Initially FIND was focussed upon diagnostics for tuberculosis (TB) (agreement with Eiken for the development of a TB LAMP kit on 29th July 2005). On 6th February 2006 FIND and WHO announced a key collaboration to improve sleeping sickness diagnostics, supported by the Gates Foundation (http://www.finddiagnostics.org/media/press/). In 2007 a LAMP reaction purported to be specific for *T. b. gambiense* was first published [141]. In 2008, FIND and their academic partners published two novel LAMP HAT assays, one for the *Trypanozoon*s, and one for the specific detection of *T. b. rhodesiense* [121, 122]. These events are summarised in the timeline shown in Figure 9.1 below.

[1] Quotation from an employee of Eiken Chemical Company in their third LAMP-related publication, within two years of initial publication of the LAMP method.

Figure 9.1. Development timeline for LAMP diagnostics for human sleeping sickness



9.2 Aims

The work described in this thesis began in September 2007 at which time a LAMP *PfrA* [140] assay and LAMP assay for *T. b. gambiense* [141] had been published. The LAMP RIME [121] and LAMP *SRA* [122] assays were published early in the course of the work. FIND was well established, but had only recently begun to apply its resources to sleeping sickness. Some LAMP tests for HAT were available, but validation work remained firmly within Phase 1 (proof of principle studies to confirm that the test detects the intended target). This study focussed initially therefore on extended Phase 1 validation and early Phase 2 work, in which the tests could begin to be applied to 'real' samples. In 2008, the publication of two novel

simple LAMP readout systems was noted, and this study was extended to include application of these systems to LAMP HAT assays. The aims of this study can be summarised as follows,

1. To determine whether LAMP assays for *Trypanozoon* detection might be a useful and simpler alternative to PCR as part of remote epidemiological surveillance studies of zoonotic parasites in the cattle reservoir.
2. To validate the published LAMP primers and assay for *T. b. rhodesiense* by comparison to the multiplex PCR for *SRA* as a reference test.
3. To design novel LAMP primers targeted to *SRA* accounting for its similarity to VSG genes.
4. To validate the published LAMP primers and assay for *T. b. gambiense* by comparison to the nested PCR for the *TgsGP* gene as a reference test.
5. To apply simple methods of LAMP endpoint detection to LAMP HAT assays.

Subsequent objectives in response to observed findings included;

6. The design and validate a novel LAMP assay for the specific detection and identification of *T. b. gambiense* by targeting the *TgsGP* gene.
7. An assessment of the reliability of LAMP assays for HAT.

9.3 Major findings and conclusions

The experimental methods and results have been described in detail in the preceding chapters. Here, the main conclusions and the results from which they are drawn are outlined and discussed.

1. Neither of the two LAMP assays for the detection of *Trypanozoon* parasites (LAMP *PfrA* designed by Kuboki *et al.* in 2003 [140] and LAMP RIME designed by Njiru *et al.* in 2008 [121]) perform as well as the equivalent PCR protocol developed by Moser *et al.* in 1989 [107]. Therefore, at present LAMP does not offer an improved system for remote epidemiological surveillance of human infective trypanosomes in the zoonotic cattle reservoir.

Cattle blood samples are routinely screened for the presence of *T. brucei* s.l. DNA, for monitoring the impact of the Stamp Out Sleeping Sickness programme (see www.stampoutsleepingsickness.com), and as part of other ongoing epidemiological studies. When the LAMP *PfrA*, LAMP RIME and TBR PCR assays were applied simultaneously to DNA eluted from cattle blood samples (stored on Whatman FTA cards), positive signals were detected in 34 % (n = 145/428) by LAMP RIME, 17 % (n = 72/428) by TBR PCR and 5 % (n = 23/428) by LAMP *PfrA*. Compared to PCR, LAMP *PfrA* was very specific (94.38 %) but insensitive (4.17 %) whereas LAMP RIME was less specific (71.63 %) but more sensitive (61.11 %). Sensitivity was also calculated for each of the three reactions compared to a cumulative reference standard.

However, subsequently the specificity of LAMP was brought into question. It is risky to compare the sensitivity of these assays to a reference standard, which does not represent a gold standard. LAMP RIME specificity was undermined when it was seen to amplify *T. congolense* and *T. vivax* control DNA. It was further undermined when a BLAST search of the LAMP primer binding region revealed a region of the *Schistosoma mansoni* genome with high sequence identity to the RIME sequence. Although this is irrelevant for screening cattle blood samples, elsewhere the LAMP RIME tool is undergoing evaluation as a human diagnostic [268]. In summary, LAMP *PfrA* is insensitive compared to TBR PCR and LAMP RIME might be non-specific. In light of these results neither of the currently available LAMP for

Trypanozoon assays is recommended as a replacement for TBR PCR for large-scale remote epidemiological surveillance of cattle blood samples.

Although in this study the LAMP assays were applied to field derived naturally exposed samples, some of which were infected and some which were not, the absolute infection status of the cattle was unverified. Although TBR PCR was applied as a reference standard, but we do not know how many true infections it might miss.

2. Both the LAMP *SRA*1 assay (designed by Njiru *et al.* in 2008 [122]) and the novel LAMP assay (designed in this study and which targets a separate region of the *SRA* gene) are sensitive and specific for *T. b. rhodesiense*.

The LAMP *SRA*1 assay was published in 2008 [122]. Using primers designed to target the *SRA* gene, the authors intended that this assay would be specific for *T. b. rhodesiense*, but had failed to account for the fact that *SRA* arose from a deletion of a 'parent' VSG gene. Since 2003 we have known that there are *VSG* genes with some sequence identity to *SRA*, but that *SRA* can be distinguished from these *VSG*s by a 378 bp deletion [173]. Picozzi *et al.* recently exploited this to create a PCR for the *SRA* gene using primers spanning the deletion site [108]. Consequently, when *T. b. rhodesiense* DNA is present, a large (>1 kb) *VSG* fragment, and a smaller (324 bp) *SRA* fragment are amplified. When *T. b. brucei* DNA is used as the reaction template only the *VSG* fragment can be seen. In this study we mapped the LAMP primers designed by Njiru *et al.* to the *SRA* gene. The primers bind upstream of the deletion site, in a region which was expected to show a high level of sequence identity to several *VSG*s. This warranted a re-validation study of the LAMP *SRA*1 assay. Surprisingly, it specifically (98%) detected *T. b. rhodesiense* when tested on 36 *SRA* PCR positive and 47 *SRA* PCR negative samples. Sensitivity was estimated at 92 %.

A novel LAMP assay (SRALAMPa) was developed, designing LAMP primers that did account for the *SRA* specific deletion site. This assay was found to exhibit 94 % sensitive and 96 % specificity.

The analytical sensitivity of both assays were similar (~ 1 pg DNA, equivalent to ten trypanosomes). Both were less sensitive than the multiplex *SRA* PCR, which can reliably differentiate between single *T. brucei* subspecies trypanosomes as well as genomic DNA diluted to the equivalent of one trypanosome [108].

Turbidity can be used to discern LAMP amplification for the novel, but not from the published assay. This might make it more amenable to a poorly-resourced, or low-technology, setting.

3. The LAMP TBG1 assay for the detection of *T. b. gambiense* (designed by Thekisoe *et al.* [141]) is not specific for this sub-species.

The LAMP TBG1 assay was developed for detection of *T. b. gambiense* in 2007 with primers targetting the 5.8S rRNA-internal transcribed spacer 2 (ITS2) gene [141]. The primers were designed using the databased AF306777 gene sequence. This spans the ITS-1, ITS-2 and intervening 5.8S rRNA of a Group 2 *T. b. gambiense* isolate known as TH2 (78E), originating from a human infection in Koudougou, Côte d'Ivoire. It was sequenced by Agbo *et al.* in 2001[249], who identified a unique four base pair C_3A insertion in the 5.8S rRNA gene of *T. b. gambiense* isolates. Aside from the LAMP TBG1 assay, this gene region has not been exploited for nucleic acid amplification based diagnosis of *T. b. gambiense*. Rather, the *TgsGP* gene is considered to be the diagnostic indicator gene for *T. b. gambiense* (Group1) [25, 109, 177]. PCR assays targeting this gene have been developed, including the nested protocol used in this study [32, 109].

In the original description of the LAMP TBG1 assay only one *T. b. rhodesiense* and one *T. b. brucei* were used to check for non-specific amplifications against with these two subspecies. This warranted a second evaluation of the LAMP TBG1 assay on a larger sample set. The assay was validated using the *TgsGP* PCR as the reference standard. Firstly, LAMP was performed in a real time turbidimeter, which incubates the reaction and provides a read out. Secondly, the reaction was incubated in a thermocycler, before the reaction products were assessed by gel electrophoresis and UV illumination. Regardless of the method used the LAMP TBG1 showed very poor agreement with the *TgsGP* PCR ($\kappa < 0.01$). Furthermore, analysis of the LAMP TBG1 primers then revealed that they did not bind to the C_3A insertion.

This assay is not fit for purpose as a *T. b. gambiense* specific assay and highlights the danger of inadequate diagnostic evaluations in which too few samples are used to check for false positive results. It also highlights how a badly chosen target can compromise the specificity of a reaction.

4. A novel LAMP assay for *T. b. gambiense*, using primers designed in this study to target the *TgsGP* gene, showed very good agreement with a nested PCR protocol for the same gene [32] and the result of the assay could be simply visualised by turbidity in the reaction tube. However, concerns remain about the reliability of the assay.

Given the poor sensitivity and specificity of the LAMP TBG1 assay for *T. b. gambiense* there remained a requirement for the design of a *T. b. gambiense* specific LAMP test. Hence, LAMP primers were designed for the *TgsGP* gene. Since similar genes have been observed in other *T. brucei* s.l. isolates with consensus to the 5' end of *TgsGP*, the LAMP primers were specifically targeted to the 3' end of this gene, in the same region as the *TgsGP* PCR primers. A protocol was developed which, when first screened using a thermocycler for incubation, followed by gel electrophoresis

and UV illumination for product detection, was highly sensitive (88.89 %) and specific (100%) compared to the *TgsGP* PCR. Positive endpoints became turbid, and this could be used as a readout system. Compared to the nested *TgsGP* PCR protocol used, the LAMP assay required a higher detection limit i.e. the *TgsGP* nested PCR is 1,000 to 10,000 times more sensitive than the LAMP reaction.

When the assay was performed in a real time turbidimeter the average time to reach the turbidity threshold was slower than had been seen with the LAMP *PfrA* reaction.

It is possible that the sensitivity and efficiency of this LAMP assay might be improved by small adjustments to the reaction composition and primer design and this assay has real potential for further development as a *T. b. gambiense* specific LAMP assay. However, the main concern is the reliability of the assay. This is discussed in more detail under point 5 below.

5. The reliability of the two LAMP *SRA* assays was imperfect, but consistently good when using purified DNAs from well characterised isolates. However, when the same assays were applied in triplicate to DNA eluted from human patient and cattle blood samples (on Whatman FTA cards) the results were less consistent. The LAMP RIME assay was also less reliable than the TBR PCR on the DNAs eluted from FTA cards.

LAMP *TgsGP* reliability was only assessed using the purified DNAs. The picture here is unclear: with a small sample set (n = 15; including 5 *T. b. gambiense*), reliability across five repeats was strong; but when the sample set was extended (n = 86, of which 9 samples were *T. b. gambiense*) agreement with the reference PCR, and in test-retest analyses was of concern.

Initially, reliability of LAMP *SRA*1, SRALAMPa and LAMP *TgsGP* were assessed by comparing three repeat screens of the 86 DNA samples used throughout the study. The published LAMP *SRA*1 (for *T. b. rhodesiense*) consistently showed very good agreement ($\kappa > 0.8$), both with the reference PCR for the *SRA* gene, and in test-retest analyses of the three repeats. The agreement between SRALAMPa (also for *T. b. rhodesiense*) and the reference PCR was slightly less strong and more varied, but did not fall below $\kappa = 0.7$. Kappa values for the test-retest reliability were also less strong and more variable with SRALAMP_a than with the LAMP *SRA*1 assay. However, even at its worst test-retest reliability did not fall below $\kappa = 0.676$, which is considered to be good agreement.

In contrast LAMP *TgsGP* (for *T. b. gambiense*) did not show consistently good agreement with the reference PCR for the *TgsGP* gene. At first agreement was very good ($\kappa = 0.935$) but at the second and third repeats this fell to $\kappa = 0.548$ and 0.554 respectively. Test-retest agreement was moderate in each instance. However this was probably skewed by the asymmetry of the sample set, which contained a low proportion of *T. b. gambiense* samples.

Next, a subset of 15 samples was subject to additional retest assays to achieve a total of five repeats for each assay. Internal agreement for the three subspecies specific assays agreement was quantified using Fleiss' kappa statistic. LAMP *SRA*1 was most reliable ($\kappa = 0.829$), then LAMP *TgsGP* ($\kappa = 0.769$), and lastly SRALAMP_a ($\kappa = 0.753$). This suggests the reliability of LAMP *TgsGP* might have been underestimated in the first analysis by the nature of the sample set.

LAMP RIME and LAMP *PfrA* were also performed in pentaplicate on this subset. The percentage agreement was better for LAMP RIME. However, this sample subset did not include non-*Trypanozoon*s, so this agreement can only be interpreted in terms of reproducible detection of true positives.

The two LAMP SRA assays and the LAMP RIME were then applied in triplicate to a set of 'field' samples (48 cattle blood samples and 52 human patient blood samples, both spotted onto Whatman FTA cards). At the same time the TBR PCR and *SRA* PCR assays were also performed in triplicate on the same DNA elutions from the same samples.

All human samples were positive by the TBR PCR as expected, and the test – retest agreement for TBR PCR on the cattle samples was very good ($\kappa > 0.8$) in each instance. By comparison the LAMP RIME was less reliable, showing only poor to fair agreement in each test –retest analysis performed on cattle and human blood samples ($\kappa = 0.024 – 0.383$).

Overall the field samples *SRA* PCR test-retest agreement was consistently good ($\kappa = 0.605 – 0.635$) and LAMP *SRA*1 showed a comparable level of test-retest agreement ($\kappa = 0.580 – 0.680$). In contrast SRALAMP_a was less reliable, with only a poor to moderate agreement in test-retest analyses ($\kappa = 0.191 – 0.443$).

Notably, the reproducibility of the LAMP *SRA* assays were worse when the field samples were used. LAMP *SRA*1 test-retest reliability ranged from $\kappa = 0.857$ to 0.880 on the control DNAs, compared to $\kappa = 0.580$ to 0.680 on the field samples. A similar pattern was seen for SRALAMP_a. The control DNAs kappa ranged from 0.676 to 0.835, whereas with the field samples kappa ranged from 0.191 to 0.443. It is possible that the reproducibility of these LAMP reactions on real samples might be improved if samples were collected, stored, and prepared for molecular analysis by another method. However, FTA cards provide an unrivalled sample collection and storage system.

So, LAMP is not always perfectly reliable and reproducibility worsens when real samples are used. However, the reliability measures here cannot necessarily be extrapolated to samples processed differently. If LAMP is developed as a clinical diagnostic, reliability must not be taken for granted but should be quantified under that system. Perfect reliability might not be a pre requisite for the application of LAMP as a clinical diagnostic, or as a research tool. For example, in a recent evaluation of a malaria rapid diagnostic test the intensity of the test line was not reproducibly read for five out of 19 samples, yet the authors concluded that it performed satisfactorily [274].

6. Several simple endpoint detection systems have been developed that might facilitate the use of LAMP assays for HAT in low-technology settings. Turbidity is the most simple, but is not possible for all LAMP HAT assays. Quant-iT PicoGreen provides an obvious colour change but is expensive, and requires that the reaction tube is opened, exposing the laboratory to contamination with the LAMP product. Calcein with $MnCl_2$ inhibits LAMP amplification and the colour change is difficult to see. Finally, hydroxynaphol blue (HNB) provides a simple, cheap, closed system, endpoint readout system. Several observers agreed that the HNB colour change was easier to see than that with calcein and $MnCl_2$. However, the agreement between the true endpoint (as determined by gel electrophoresis) and the HNB endpoint was variable for different observers. Further work is required to develop methods that will assist different observers to make consistent interpretations of the same colour change.

Several simple endpoint detection methods have been developed for LAMP. In this study four methods were applied and compared for LAMP HAT assays: (i) visualization of turbidity; (ii) hydroxynaphthol blue (HNB) added before incubation; (iii) calcein with $MnCl_2$ added before incubation and (iv) Quant-iT PicoGreen added after incubation. Quant-iT PicoGreen was the most expensive of the methods used

costing £232.00 for 100 reactions (using 5 µl per reaction). The other methods all cost less than £0.001 for 100 reactions.

Turbidity and the metal ion indicator methods (HNB and calcein) are the simplest readout systems to have been developed. Turbidity requires no additional reagents or technology. The metal ion indicators are added with the other reagents in the mastermix before incubation. For both, amplification and detection are combined in single processing step, within a closed tube system. The readout can be made by eye, without special lighting, and they are inexpensive.

Turbidity is not visible for the LAMP RIME or *SRA1* assays using published protocols. Both of these assays could be adjusted to obtain turbidity by simultaneous increase of $MgSO_4$ and dNTPs. However, this has unknown consequences for the diagnostic sensitivity and specificity of these assays.

Generally, when turbidity was compared to the true endpoint (as determined by gel electrophoresis) for LAMP *PfrA*, *SRALAMP_a* and *TgsGP* the positive predictive value of a turbid result was very high, but the negative predictive value was lower and more variable. Agreement, quantified by Cohen's kappa statistic, typically fell between 0.7 and 0.8, indicating good agreement. Clearly however, this agreement is imperfect and it varied for the three repeats of each assay on the same samples. This compromising variation was observed by a single reader, but it is not clear whether this is intra-reader variability or intrinsic variability of turbidity.

A multi-observer study with 33 participants was used to investigate the reliability of the two metal ion indicator methods used in LAMP diagnostics. These methods were chosen as low cost, closed systems (c.f. Quant-iT PicoGreen). Turbidity was excluded because it is not visible for all HAT LAMP assays.

Hydroxynaphthol blue was the better of the two metal ion indicator methods tested. All observers said they found the hydroxynaphthol blue colour change easier to see. It showed better inter-reader reliability and more consistent agreement with the presence of the DNA amplicon (as determined by gel electrophoresis).

However, Cohen's kappa statistic for the agreement between the colour change (interpreted by a single observer) and the true endpoint (determined by gel electrophoresis) ranged from $\kappa = 0.145$ to $\kappa = 0.870$ depending upon the observer. Therefore if several people are involved in LAMP screening, training and quality control will be necessary to reduce inter-observer variation. This process might be aided by generating a colour swatch card against which assay results can be compared, along the lines of a litmus paper test for pH. Better still, a quantitative colour measure, using some kind of spectrophotometric device would remove this variability, although we must not forget that we are seeking to establish a LAMP endpoint detection format that is suitable in a low resource setting.

Finally, it is critical to bear in mind that all the above methods are incapable of verifying that the LAMP product has been amplified from the intended target. For this, more complex approaches are required, such as sequencing. This issue has been discussed above in relation to the LAMP RIME assay.

9.4 Further work

9.4.1 Arising from Chapter 3

Given the observed LAMP RIME non-specificity (performed on only two non-*Trypanozoon* control DNAs, and two commercially available host genomic DNAs) it would be useful to perform an extended Phase 1 proof of principle trial to confirm, with finality, the specificity or otherwise of LAMP RIME. In this study we moved

directly to a Phase 2 evaluation of LAMP RIME and LAMP *PfrA*, since Phase 1 appeared to have been completed in the original publications. An extended Phase 1 proof of principle study ought to include more non-*Trypanozoon* DNAs. In the original study 13 non-*Trypanozoon* DNAs were used. No false positives were reported, corresponding to 100 % specificity (95 % confidence interval 73.41 – 100 %). Table 9.1 below shows how this confidence interval might be narrowed by choosing a larger sample of non-*Trypanozoon*s.

Table 9.1. The effect of sample size on the confidence interval of a specificity measure

Sample size	Specificity (95 % CI)		
	0 false positives	**1 false positive**	**All false positive**
13	100 % (73.41 – 100 %)	92.31 % (64.58 – 99.99 %)	0 % (0 – 26.59 %)
30	100 % (86.53 – 100 %)	96.67 % (81.91 – 99.99 %)	0 % (0 – 13.47 %)
50	100 % (91.48 – 100 %)	98.00 % (88.53 – 99.99 %)	0 % (0 – 8.52 %)
100	100 % (95.56 – 100 %)	99.00 % (94.01 – 99.99 %)	0 % (0 – 4.44 %)

Other useful further work to investigate the observed non- specificity could include:

(i) sequencing of LAMP RIME products where they arise from non-*Trypanozoon* control DNA samples;

(ii) sequencing of LAMP RIME products where they arise from simultaneously TBR PCR negative cattle blood samples;

(iii) restriction digest of LAMP RIME products (using *Nde*I which cuts between primers F1c and B1 [121]) where they arise from non-*Trypanozoon* control DNA samples;

(iv) restriction digest LAMP RIME products (using *Nde*I which cuts between primers F1c and B1 [121]) where they arise from simultaneously TBR PCR negative cattle blood samples;

(v) application of LAMP RIME to *Schistosoma mansoni* DNA.

If LAMP RIME is validated as a specific assay, or a novel, *Trypanozoon* specific LAMP assay is developed which passes Phase 1 validation, then it would be useful to assess the assay using a no gold standard approach. No gold standard approaches are described in more detail in the supplement. In short, several frequentist and Bayesian latent class analysis methods have been developed to estimate the sensitivity and specificity of two or more index tests, in the absence of a gold standard. These approaches require that the tests are applied in more than one population.

9.4.2 Arising from Chapter 4

The next step for the *SRA* LAMP assays is Phase 2 evaluation. Phase 2 evaluation involves a case control study, ideally using fresh specimens. Archived samples can be used for Phase 2 trials, which may be quicker, cheaper and more convenient. However, the specimen quality may have been affected by storage, patient information may be limited and consent might be problematic [267, 275].

Very recently a study was published in which LAMP *SRA*1 and LAMP RIME were compared to a PCR for the *SRA* gene using microscopy positive patient blood spotted onto FTA cards [268]. The PCR [231] against which the LAMP assays were compared was a nested protocol with different primers to the multiplex *SRA* PCR used throughout this study. This was a useful study, demonstrating that LAMP *SRA*1 was 93.8 % sensitive (95 % CI 88.2 – 96.8 %) overall, and was significantly more sensitive that the PCR protocol. However, the analytical sensitivity of this particular PCR has not been reported in the literature. This highlights a more general problem: LAMP is rightly being compared to PCR, and yet, different studies are basing their comparisons on different PCR protocols. As has been pointed out elsewhere there is a plethora of in-house variations for any trypanosome PCR [232]. Ideally, before more authors set out to perform comparisons of the LAMP *SRA* assays to PCR, a

standard protocol ought to be agreed. Further, it would be very useful to have a standard specimen bank on which comparisons are made [232].

Returning to Phase 2 case control evaluation of the LAMP *SRA* assays, we face the problem of a reference standard. If LAMP is truly more sensitive than any previous parasitological detection method, then LAMP might detect true, but undetected, cases from within the control group. The TBR PCR could be the most appropriate reference standard, since it has a multi copy DNA target, making it a more sensitive target than the single copy *SRA* gene. However, the TBR PCR might detect DNA from abortive [91, 92] and/or transient [39] *T. b. brucei* infections. (This is discussed in more detail below in regard to the clinical significance of detecting trypanosome DNA.) Methods for diagnostic evaluation in the absence of a gold standard are also available and might be more appropriate. These are discussed in more detail in the supplement to this chapter. Notably, they require a different study design to the traditional case control study. In particular, they require that the index tests are compared in more than one population, which have different prevalences of disease.

Prior to Phase 2 evaluation DNA sample preparation protocols need to be developed and properly validated. In the study discussed above patient samples from blood spots on FTA cards were used. This format is useful for remote epidemiological studies, but might be less appropriate if LAMP is to be applied at point-of-care. Elsewhere LAMP *PfrA* has been trialled using various DNA templates, including (i) genomic DNA extracted by the phenol-chloroform method; (ii) fresh blood applied directly to the reaction, (iii) haemolysed blood (prepared by dilution in distilled water, followed by centrifugation and re-suspension of the precipitate in TE buffer) and (iv) DNA eluted from FTA card samples. LAMP *PfrA* amplification sensitivity was consistently high (100 %) for serially diluted DNA (1000 pg to 1 pg) for all of the above DNA templates except fresh blood, for which the detection sensitivity fell as low as 43 % [230]. In another study heat treated blood samples were successfully used to seed a LAMP assay for *Plasmodium falciparum* [126]. The template

preparation protocol must also be designed to be compatible with the endpoint detection format. Thekisoe *et al.* rightly point out that turbidity cannot be visualised when fresh or haemolysed blood is used, and is difficult when FTA card eluate is used [230]. In this study two colour change methods for LAMP endpoint detection were trialled using FTA card eluate (Chapter 8 and discussed below).

In summary, validation of the LAMP *SRA* assays is ready to move to Phase 2. Further work may need to be done to validate particular template preparation and endpoint detection formats in themselves before they are incorporated into a LAMP *SRA* Phase 2 trial. What is more, several different Phase 2 studies may need to be performed to validate different applications of the assay, which will likely use different sample preparation and endpoint detection formats (e.g. a case control study as a clinical diagnostic versus a case control study as remote epidemiological surveillance tool). It is critical that the objectives of any validation study are clearly defined from the outset. Sensitivity and specificity values cannot properly be extrapolated to include a different test protocol or application.

9.4.3 Arising from Chapters 5 and 6

Firstly, it might be possible to further optimize the primer design, reaction composition and incubation conditions in order to improve the sensitivity and efficiency of this assay.

Secondly, it would be extremely useful to extend the validation across a much larger set of well characterised *T. b. gambiense* isolates from across the endemic range for this disease. In this study the novel LAMP *TgsGP* was validated on a relatively limited collection of available *T. b. gambiense* isolates (nine *TgsGP* PCR positive samples). Earlier, the effect of sample size on the confidence limits surrounding specificity estimates was discussed. The same principle applies to sensitivity (Table 9.2.). It is clear that as the number of true positives in the sample increases the 95 %

confidence interval around the sensitivity estimate decreases. For example, if one false negative result is seen with nine true positives (in this study nine *TgsGP* PCR positive samples were used) the confidence interval ranges from 60.63 to 99.17 %. If the number of true positives were to be increased to 30, the breadth of the confidence interval would be more than halved (for the same 1 false positive).

Table 9.2. The effect of sample size on sensitivity and specificity estimates

Sample size (of true positives for sensitivity, or true negatives for specificity)	Sensitivity (with 1 false negative) or Specificity (with 1 false positive)	95 % Confidence Interval
9	88.89 %	60.63 % - 99.17 %
30	96.67 %	81.91 % - 99.99 %
50	98 %	88.53 % - 99.99 %
100	99 %	94.01 % - 99.99 %

Thirdly, the next step is to apply this assay to clinical samples, in a Phase 2 case control evaluation as described for the *SRA* LAMP assays above. All of the points made above regarding clinical evaluation of the *SRA* LAMP assays are pertinent here, including considerations of how best to evaluate a diagnostic in the absence of a gold standard. However, Phase 2 evaluation will not be appropriate until a more comprehensive Phase 1 evaluation has been completed.

In addition there remains a diagnostic gap for Group 2 *T. b. gambiense*, which is not detected by the *TgsGP* PCR assay, nor this newly developed LAMP assay.

9.4.4 Arising from Chapter 7

The reliability of any diagnostic is critical and the results in Chapter 7 are concerning. Of particular concern are (i) the reliability of the *TgsGP* LAMP on the purified DNA extractions, and (ii) the reduction in reliability seen when DNA is eluted from FTA card samples.

As mentioned above, it would be useful to extend the set of DNA samples to include more *T. b. gambiense* isolates. This would improve the precision of the accuracy estimates for LAMP *TgsGP* (see future work arising from Chapters 5 and 6) as well as improving reliability estimation here.

It would be useful to add endemic and non-endemic human control samples to this investigation of LAMP *SRA* reproducibility on human blood samples. It would also be natural to continue the assessment of LAMP *TgsGP* with a similar reliability study using human case and control blood samples.

Similarly, LAMP RIME and LAMP *PfrA* reliability assessment would also be improved with additional testing on non-*Trypanozoon* control DNAs, as well as blood sample negative controls.

Finally the reliability of LAMP using different DNA extraction formats would be useful. It might be that LAMP is inherently unreliable when applied to DNA eluted from FTA cards. It would be useful to ascertain whether LAMP is more reliable using, for example, haemolysed or heat treated blood samples, especially since these formats have been shown to provide a suitable LAMP template in other studies [126, 230].

9.4.5 Arising from Chapter 8

In order to improve LAMP readout in resource poor settings it would be useful to develop low technology methods to reduce the subjectivity of colour and turbidity assessment. This could include a colour matching chart for on site interpretation. Martinez *et al.* [276, 277] have considered the same problem (how to obtain diagnostically useful information in remote settings from subjective colorimetric assays). They proposed a telemedicine system in which the technician performs a basic test, photographs the results using a cell phone camera, and transmits the image trained medical professional. The diagnosis/ treatment and management protocol is then returned directly to the technician/ healthcare provider. Recent studies have successfully demonstrated that camera phones can be used to transmit diagnostic images for remote interpretations [278, 279]. This approach is appealing since phones, including camera phones, are becoming increasingly common. In 2009 the percentage of mobile phone subscriptions per 100 inhabitants rose to 57.9% in the developing world (http://www.itu.int/ITU-D/ict/statistics/material/graphs/Mobile_cellular_98-08.jpg). However, the image itself will vary according to the phone itself, and the lighting conditions. This might be overcome in part by including a calibration chart into the photograph or with the development of more complex software for automatic image calibration. This approach might be useful for sleeping sickness, since treatment and management is not straightforward.

9.5 LAMP as a future clinical diagnostic for sleeping sickness?

Thus far the history of LAMP for sleeping sickness and the advances made in this study have been described. It is clear that LAMP has potential as a novel molecular diagnostic for HAT. However, it is much less clear whether this will translate into a field friendly clinical diagnostic, or whether LAMP will have a more restricted role as an (albeit useful) addition to an ever expanding tool box of molecular assays for use in sleeping sickness research. To address this question this discussion will move on to consider: (i) the political and economic factors shaping the drive to generate a

LAMP diagnostic for sleeping sickness; (ii) the operational feasibility of LAMP; (iii) competitors to LAMP and (iv) the clinical significance of identifying parasite DNA.

9.5.1 Politics and economics

In his book, 'African Sleeping Sickness: Political Ecology, Colonialism, and Control in Uganda (Studies in African Health and Medicine)' Jonathan Musere states

'It is not just enough to solely examine the scientific and medical basis of a disease, since in many cases the socio-political basis is the most important.'[280].

Further, the development of loop-mediated isothermal amplification (LAMP) based diagnostics for sleeping sickness must be considered within historical, political and economic context under which they have been, and continue to be, developed. Biological factors are not alone in shaping the development of diagnostics.

Sleeping sickness is a neglected tropical disease, but this has not always been the case. It has also been described as 'the first media event epidemic in modern Africa' [281]. In the early colonial period massive sleeping sickness epidemics swept across East and West Africa. The epidemics terrified the colonial powers and stimulated huge control programmes: as a consequence transmission was almost halted by the 1960s. Yet in the post colonial era cases once again began to rise; by the late 1990s incidence had returned to levels seen in 1930s [59]. This resurgence has been attributed to declining surveillance [59] as well as to socio-economic upheaval [282]. And so, human African trypanosomiasis became a neglected tropical disease.

The so called 'neglected tropical diseases' affect more than one billion people, and persist under conditions of poverty. They are neglected at all levels. At the community level the marginalised are often most affected and stigma keeps the

diseases hidden. The diseases themselves are mostly low mortality, high morbidity diseases, which cause severe and permanent disability. Consequently, at the national level, limited resources are allocated instead to high mortality, high priority diseases such as HIV and tuberculosis. At the international level they receive relatively little attention because they do not pose an immediate threat to western societies [60].

In 1995 the World Health Organisation began to express concern about the rising incidence of sleeping sickness. In 1997 they passed a resolution in which they strongly advocated access to diagnosis and treatment and the reinforcement of surveillance and control [283]. According to Simarro *et al.* [59] there have been widespread improvements in HAT control since 1995 including technical and financial support from the WHO and high level political will which have increased the capacity for control. At the same time, access to diagnosis and treatment have been facilitated by declining social upheaval and an end to civil war in many endemic regions. In addition a free and sustainable drug supply was secured from industry. However, it was impossible to extend the provision of free drugs to include diagnostics, because commercially available diagnostics did not (and still do not) exist. Indeed, no diagnostic test for HAT has ever been manufactured under full registration by any regulatory agency and the tests that are currently available are produced by academic institutions [268].

To address this problem the Foundation for Innovative New Diagnostics (FIND; see www.finddiagnostics.org) was launched on 22nd May 2003 at the World Health Assembly as a spin off from the World Health Organisation (WHO). It is a non profit, Swiss-based foundation which 'supports the development of diagnostic tests for diseases of poverty, including TB, HAT and malaria.'[268]. FIND have adopted a 'piggy backing' approach: by developing technology platforms that are applicable to multiple diseases they have persuaded technology development companies to include neglected infectious diseases into diagnostic platforms for more commercially attractive diseases [284]. For example, FIND have developed a light-emitting diode

(LED) based fluorescence microscope with their industrial partner Zeiss. This technology was first developed to improve microscopy for TB, and then was applied to parasite detection for sleeping sickness. LAMP is the second example of FIND's piggy backing approach to develop diagnostics for sleeping sickness. As described in Chapter 1, the LAMP technology was developed by Eiken Chemical Company in Japan [123]. Together FIND and Eiken developed and tested a prototype kit for LAMP based TB diagnosis using sputum samples [135] (also described in Chapter 1). On 27th October 2008 FIND and Eiken announced their intention to extend this collaboration to work on HIV, malaria and sleeping sickness (http://www.finddiagnostics.org/media/press/081027.html). In the meantime FIND, working with academic partners at Murdoch and Obihiro Universities, developed the LAMP RIME and LAMP *SRA* assays for the detection of *Trypanozoon* and *T. b. rhodesiense* parasites respectively. Hence, LAMP was applied to sleeping sickness, because of a pre-existing collaboration with a particular technology development company. This approach was necessitated by the neglected status of human African trypanosomiasis, which renders it commercially unattractive. As stated elsewhere, '*The development of new diagnostic tools has been underfunded largely because neglected tropical diseases do not represent a significant market.*' [60].

HAT is now at its lowest level for 50 years and we are experiencing a long inter-epidemic period. Only 9877 new cases were reported in 2009 (http://www.who.int/neglected_diseases/integrated_media/integrated_media_hat_june_2010/en/index.html). However, these case reports are an underestimation of the true extent of sleeping sickness as under reporting is a known concern and it is possible (as suggested in Section 1.5.5 below), that latent *T. b. gambiense* may be circulating in unidentified foci. These figures are extremely low in comparison to other diseases such as malaria and tuberculosis, reinforcing how financially unattractive the development of HAT diagnostics are. In 2008, there were 9.4 million new cases of tuberculosis (http://www.who.int/tb/publications/2009/tbfactsheet_2009update_one_page.pdf)

and 247 million cases of malaria causing nearly 1 million deaths (http://www.who.int/mediacentre/factsheets/fs094/en/index.html). Furthermore, as case numbers fall HAT diagnostics become less and less commercially viable.

9.5.2 Operational feasibility

As outlined in Chapter 1 there has been an explosion of LAMP research publications describing its application as a molecular diagnostic for a wide range of pathogenic agents. The original publication has been cited 317 times (on July 21st 2010), including 274 articles, 31 reviews, nine proceedings papers, two letters and one editorial. In addition several commercial kits have been developed by Eiken Chemical Company (see http://loopamp.eiken.co.jp/e/products/index.html), some of which have been adopted as the officially recommended methods for routine identification and surveillance of pathogens in Japan [138].

With Public Private Partnerships come Public Private rivalries and conflicts and interests. According to Mori and Notomi (key players in the development of LAMP and employees of Eiken Chemical Company);

'*the current direction of the development of LAMP is toward a simple diagnostic tool which can be routinely employed in the poorly resourced laboratories in rural areas of developing countries*' [138]

FIND and Eiken together have developed and tested a prototype LAMP kit for the detection of TB from sputum smear samples in microscopy centres of developing countries [135]. Overall the study results were positive. After one week of training, technicians with no prior experience of molecular work were able to use the LAMP kit with a mean hands-on time of 54 minutes and with excellent inter-reader reliability, in a simple laboratory environment. The technicians were reported to have

been very satisfied with the interpretation of the LAMP result (using calcein as a colour change indicator) and sample processing was considered to be comparable to the process required for culture. However, the process required multiple manipulations and a specially designed heating and observation block. Furthermore, the technicians had reservations that it could be rolled out to more peripheral facilties. There was concern that the process might be too complex for less motivated and skilled staff, and that the LAMP process cannot be interrupted, for example, during a power cut, as can microscopy. Following this study LAMP and Eiken agreed to continue the development and simplification of the kit, and to extend performance testing.

Others disagree that LAMP might be a useful tool in remote rural healthcare settings. Referring to the use of LAMP for sleeping sickness diagnosis, Deborggraeve and Büscher recently stated that;

'*LAMP is being presented as a promising technique for point-of-care diagnosis of sleeping sickness. However, one should realise that diagnosis of this disease is most often done in very remote areas devoid of even basic facilities. LAMP requires DNA extraction, electricity, a cold chain, multiple manipulations, and sufficient infrastructural measures to prevent carry-over contamination. Therefore, we are not convinced of its future as a point-of-care diagnostic test.*' [232][2]

Deborrgraeve and Büscher highlight several important concerns about the use of LAMP, in its current research format, for application as a clinical diagnostic in sleeping sickness endemic areas. At present LAMP for HAT requires two heating blocks (one for the incubation at 60-65 ˚C, and one for the termination step), which

[2] Notably, Deborggraeve and Busher work at the Institute of Tropical Medicine, Antwerp, where the exisiting diagnostic test that is supported and purchased by the WHO, the CATT test, was developed and is produced.

requires an electricity supply. The preparation of the reaction mix requires accurately calibrated pipettes, and disposable plastic ware (including pipette tips, PCR tubes, and e.g. an eppendorf tubes in which to make the master mix). The reagents themselves (*Bst* DNA polymerase, reaction buffer, $MgSO_4$, betaine, dNTPs and primers) are also needed, though Thekisoe *et al.* have demonstrated that it might not be necessary to maintain a strict cold chain for their storage [230]. This is a long way from the simplicity of, for example, the rapid diagnostic tests for malaria (lateral flow dipstick tests which typically require a finger prick blood sample, and provide a diagnosis in 10-15 minutes).

However, it would be short-sighted not to consider the ways in which the LAMP technology might be developed and simplified to make clinical diagnoses in resource poor settings possible. For example, the LAMP for TB prototype kit utilised a lyophilised reaction mix, which included the *Bst* polymerase. This both simplified the process (it was not necessary to accurately measure several small volumes of different reagents while keeping the whole mixture on ice) and overcame the need for a robust cold chain.

Several other developments have been reported in the literature (and it is likely that given the commercial nature of LAMP other developments are being held firmly under wraps prior to patent applications). For example, a novel integrated isothermal device for real time turbidity measurement has been developed [180]. At present this system is only capable of processing one sample at a time, however the authors '*hope to provide a multi-channel, portable, label-free, real time monitoring device for rapid identification and quantification of pathogenic organisms and point of care applications*' in the future. In a very different development LAMP and subsequent ultrafast electrophoretic analysis of the LAMP product have been integrated on a commercially available polymer biochip. However, at present this chip needs to be placed in a incubator for amplification and in a separate instrument for electrophoresis [285].

Since 2008 ten articles have been published in which lateral flow dipsticks (LFDs) have been developed for the detection of LAMP products. Recent pertinent examples include the development of LAMP-LFDs for *Trypanosoma evansi* strain B [286] and African Swine Fever virus [287]. However, while LFDs might offer a useful format for LAMP endpoint detection, they do not simplify the LAMP amplification process in itself. Rather they add manipulation steps, and the LAMP reaction tube must be opened, exposing the laboratory to amplicon contamination.

9.5.3 A second isothermal nucleic acid amplification test for HAT

Nucleic acid sequence based amplification (NASBA) is the second isothermal amplification technique to have been applied to sleeping sickness [145]. The assay, which targets the multicopy 18S rRNA, detects both *T. b. rhodesiense* and *T. b. gambiense*, but does not distinguish between the two. After an initial 2 min step heating step at 65 ˚C, the reaction is then held at 41˚C for 90 min which is more time consuming than LAMP. The assays had a detection limit of 10 parasites ml-1 which is comparable to the LAMP SRA assays. Unfortunately, cross reactivity against the Trypanosome subgenera *Nannomonas* and *Duttonella* were not assessed. Therefore this assay cannot be used for the specific detection of *T. brucei* s.l. in livestock, in which *T. congolense* and *T. vivax* also circulate in abundance, without further validation. The assay was tested on 59 blood samples from confirmed HAT cases, and 50 from controls and detected *T. brucei* s.l. in significantly more patient samples than microscopy alone.

In 2009 this assay was coupled to an oligochromatographic dipstick detection format (NASBA-OC). Coloured lines appear, in the presence of amplified product, within 5 -10 minutes of application to the dipstick, without compromising reaction sensitivity or specificity [146]. NASBA-OC was compared to a composite reference standard

(microcopy, microhaematocrit centrifugation with microscopy, or mAECT with microscopy) on 122 blood and CSF samples, and samples from Uganda (*T. b. rhodesiense*) and the Democratic Republic of Congo (*T. b. gambiense*). In brief, NASBA-OC was more sensitive than standard microscopy for blood samples but not for CSF. However, NASBA-OC requires less CSF than microscopy.

In a Phase 2 case control study [288] NASBA-OC was more sensitive and specific than PCR-OC for the same gene [289]. However at a country level the data presented a more complex picture: the specificity of PCR-OC and the sensitivity of NASBA-OC were both significantly higher in the Democratic Republic of Congo than Uganda which might be due to differences in sample transportation and storage conditions.

Deborggraeve and Büscher raise similar concerns about the applicability of NASBA-OC in a clinical setting as they did for LAMP.

'*Although such strategies will definitely facilitate the implementation of molecular diagnostics in several control strategies for sleeping sickness, they are still too complex for general use in first-line diagnosis and management of patients*.'[232]

9.5.4 Other diagnostics for sleeping sickness

Molecular techniques that do not include an amplification stage have also been proposed for sleeping sickness diagnosis, and it has been suggested that they might be more amenable to low-resource settings than the amplification based methods [232]. Two methods have been highlighted. First, Radwanska *et al.* developed a direct detection method using fluorescence in situ hybridization (FISH) with peptide nucleic acid probes [290]. The development of battery powered LED based fluorescence microscopes, by FIND and others, could make this a more field friendly

technique [232]. Secondly, Lorger *et al.* proposed a method of parasite visualization using RNA aptamers directed to the parasites surface coat [291]. However, for this technique to be sensitive a parasite concentration step would be required, such as microhaematocrit centrifugation for microscopy.

Aside from molecular diagnostics, advances are also being sought, particularly by FIND, for better microscopic parasite detection, serodiagnosis and disease staging tools (http://www.finddiagnostics.org/programs/hat/find_activities/index.html).

To improve direct parasite detection FIND have developed a simple LED based fluorescence microscope (with Carl Zeiss) using acridine orange for parasite staining. They are also improving the materials used in mAECT kits, particularly the gel columns, collection tubes, and viewing chambers, and to improve availability and procurement for screening programmes. Finally, they have revived an old method using ammonium chloride for red blood cell lysis prior to centrifugation which might be useful for parasite concentration.

Following work done by FIND and their partners to identify candidate antigens for detection of anti- trypanosome antibodies in patient sera, FIND have recently signed an agreement with Standard Diagnostics Inc., Korea for the commercial development of rapid, point-of-care test. The test will have a lateral flow format for detection of *T. b. rhodesiense* and *T. b. gambiense* (but will not discriminate between these two parasites), and is intended for screening in remote settings.

In addition FIND are collaborating with the University of Brussels on the feasibility of camel heavy chain antibodies for parasite antigen detection, and with the Seattle Biomedical Research Institute to apply single chain variable fragment (scFv) antibody engineering to developed antibody probes.

Since the lumbar puncture (currently required to obtain CSF for disease staging) is painful and risky, FIND and their partners have sought blood based biomarkers for to discern early and late stage disease, but with little success. However, they have determined a panel of three brain damage marker proteins which can be used to discriminate stage 2, and could be of use in developing improved CSF staging tests [113]. FIND have also been involved in reformatting the latex/IgM card agglutination test into a more stable individual kit format for staging by CSF IgM quantification.

9.5.5 Clinical significance of the detection of trypanosome DNA?

So far we have considered the accuracy and reliability of LAMP assays for HAT. We have also discussed the operational feasibility of LAMP in a clinical setting. Yet we must also take a step back and ask 'What is the clinical significance of the presence of parasitic DNA?'

This is a particularly pertinent question for diagnosis of *T. b. gambiense*, for which discrepancy between PCR and other diagnostic indicators have been previously noted [91, 92, 244, 292].

An investigation into a potential new focus of *T. b. gambiense* provides an excellent example of this uncertainty [292]. In late 2007 PCR analysis of human blood samples, from a previously 'silent' region around Lake Albert in western Uganda, generated positive signals for *T. b. gambiense*. Active screening (by CATT) was initiated in September 2008. All CATT whole blood positive individuals provided a second blood sample for CATT on 1/4, 1/8 and 1/16 plasma dilutions and for parasite detection by micro-haematocrit centrifugation and were examined for clinical signs by cervical lymph node palpitation. CATT positive whole blood was also spotted

onto Whatman FTA cards for PCR analysis (using both TBR PCR and *TgsGP* PCR for *T. brucei* s.l. and *T. b. gambiense* respectively) in the UK.

Parasites were not found in the blood of any of the screened individuals by micro-haematocrit centrifugation, nor did any of the individuals examined show enlarged lymph nodes. However several individuals were serologically positive by the CATT test (down to 1/16 plasma dilution) and several of the blood samples were positive for *T. brucei* s.l. parasite DNA by PCR. These were confirmed as *T. b. gambiense* in a sub set of samples. CATT seropositivity and PCR positivity did not agree. A follow up study of CATT whole blood positive cases was performed six months later, in which CATT was repeated, and the more sensitive mini-anion exchange centrifugation technique (mAECT) was used to detect parasites. Again, PCR positives were obtained, yet no parasites could be detected. The epidemiological picture became even more peculiar when these CATT and PCR results did not match those seen in the initial screening phase.

Koffi *et al.* [92] noted similar discrepancies for TBR PCR compared both to CATT seropositivity and parasitological detection by mAECT in Côte d'Ivoire. Observing that these disagreements were worst among aparasitaemic serological subjects, they concluded that there might be a long lasting human reservoir of *T. b. gambiense*. The evidence for spontaneous cure and chronic state for *T. b. gambiense* has been carefully reviewed elsewhere [45]. These authors cautiously conclude that, while chromic carriage is biologically plausible, there is no current evidence that it does occur, but that there is some evidence for spontaneous cure from early stage disease.

So, the questions remain

What is the clinical significance of detecting parasite nucleic acids?

Are we detecting pre-patent or transient T. b. gambiense infection?

Is this infection with non-pathogenic strains of T. b. gambiense?

Are we detecting chronic carriage in trypanotolerant individuals?

At present, we do not know the clinical significance of parasite DNA detection. If there are instances of human trypanotolerance, and/or non pathogenic *T. b. gambiense* strains in circulation, then the current epidemiological paradigm would need to be reworked with major implications for long term planning of disease control programmes. Further, Koffi *et al.* [92] note that the TBR PCR might be detecting persistent DNA from abortive *T. b. brucei*. infections, a suggestion that has also been made by Garcia *et al.* [91]. More recently transient *T. b. brucei* infection has also been demonstrated [39]. Hence, molecular assays for detection of *Trypanozoon* DNA, including LAMP [121, 140] and NASBA [146] as well as traditional [107] and real time PCR [186], need to be treated with caution. On the other hand, while multicopy *T. b. gambiense* and *T. b. rhodesiense* diagnostic indicator genes remain unavailable, multicopy *Trypanozoon* genes remain the most sensitive molecular targets for these trypanosomes.

Deborggraeve and Buscher [232] suggest that *T. brucei* nucleic acids might be a marker for infection, but not disease, as has been observed for a PCR based Leishmania diagnostic [293]. They also suggest that *T. brucei* DNA might even be incorporated into the human genome giving rise to false positive PCR results.

9.6 Evaluating diagnostics in the absence of a gold standard

Ideally new diagnostics are evaluated by comparison to a perfect reference test. However, often such a 'gold standard' may be unavailable for some or all of the samples, or may be imperfect or in some cases does not exist [294]. This leads to classification errors, which if ignored could bias accuracy estimates for the index test [295]. Consequently, latent class analysis methods have been developed to estimate

the sensitivity and specificity of two or more index tests, in the absence of a gold standard. Latent class methods have been used for more than 30 years [296, 297]. Both frequentist and Bayesian statistical approaches have been developed for latent class analysis and are outlined below.

9.6.1 Frequentist approaches

Hui and Walter [298] were the first to describe maximum likelihood analyses for latent class models. According to the Hui Walter paradigm two or more populations, with different disease prevalence in each population, are required to assess two tests. The properties of each test must be constant across these populations, and the tests must be conditionally independent given the true disease status. These assumptions are necessary to ensure that that the degrees of freedom of the observed data exceed the number of parameters to be estimated, i.e. to make the problem statistically identifiable [299]. Other frequentist approaches have also been described which make use of maximum likelihood methods for latent class analysis [300, 301]. All maximum likelihood based approaches require a large number of observations, since maximum likelihood theory is based on large sample theory. An excel based format to facilitate sample size calculations has been developed and is available online (www.epi.ucdavis.edu/diagnostictests) [302].

9.6.1.1 A user friendly latent class analysis tool

'TAGS', a program for the evaluation of test accuracy in the absence of a gold standard, was developed to promote the application of a maximum likelihood method to latent class analysis in a user friendly format. The programme can be implemented in the R and S-Plus statistical packages, and is also available in a web based interface, which in turn submits data to R. TAGS can be used with any statistically identifiable data set; data can be incorporated from several populations and multiple tests. Data from a reference population in which the true status is known can also be accounted for [303].

9.6.2 Bayesian models

Bayesian models have also been developed for latent class based diagnostic evaluation where there is no gold standard. The Bayesian approach allows prior scientific information to be incorporated into estimations of test accuracy and to solve otherwise statistically unidentifiable problems.

Several Bayesian models for sensitivity and specificity estimation have been described:

i) One test in one population
ii) Two conditionally independent tests in two or more populations
iii) Two correlated tests in two or more populations
iv) Three tests in two or more populations, where two tests are correlated but jointly independent of the third test [304].

Some have questioned the legitimacy of Bayesian approaches to solve statistically unidentifiable problems. Particularly, how is it possible to obtain prior sensitivity and specificity estimates if there is no gold standard [299]?

Recently a Bayesian formulation of the Hui-Walter latent class model has been used to compare two PCR based molecular detection assays for *T. brucei* s.l. [305]. Given the limited sensitivity of current parasitogical detection methods a no gold standard approach might be best for evaluating LAMP in a clinical setting. This would require a very different study design to a traditional Phase 2, case-control evaluation.

9.6.3 Limitations of latent class analysis

In latent class models, individuals that are not detected by any of the available tests are assumed to be uninfected. This is an important limitation to all latent class models that has not been well addressed in the literature. In order to avoid this problem it is important to use a range of tests covering the spectrum of biological responses to infection. If this is not done, a group of infections may be missed, the sensitivity of other tests will be overestimated, and true prevalence underestimated [297, 303].

9.7 Final words

Numerous sensitive and specific molecular diagnostics have been developed for sleeping sickness [232]. They are invaluable as research tools and PCR is routinely applied in our laboratory for surveillance of *T. b. rhodesiense* in cattle, however molecular tools have not been adapted for clinical use, and do not directly benefit sleeping sickness patients. But now, LAMP is being developed and promoted as molecular diagnostic for clinical application and FIND have signed an agreement with Eiken Chemical Company to develop LAMP for sleeping sickness.

In this study the published LAMP assays for *Trypanozoon* were evaluated for surveillance of *T. brucei* s.l. in cattle, and determined that they are unsuitable replacements for the current, well-established PCR protocol. There remains scope for the development of an improved LAMP assay for *Trypanozoon* detection. The published *T. b. rhodesiense* - and *T. b. gambiense*-LAMP assays were also evaluated, in addition to designing novel assays for both these subspecies.

Contrary to expectation (given the primer design), the published LAMP for *T. b. rhodesiense* was sensitive, specific and reliable for purified DNAs, as was the novel assay. The novel *T. b. gambiense* LAMP (which targets the *TgsGP* gene), but not the

published assay (which targets the 5.8S rRNA gene) was sensitive and specific. However, the reliability of LAMP *TgsGP*, may be less than optimal and requires further assessment on an extended sample set.

In summary this work has highlighted problems with some of the published LAMP assays for the detection of sleeping sickness parasites and, where possible, improved LAMP assays have been developed in their place. This work also emphasizes the importance of simple readout for LAMP tests, and evaluated several of the currently available methods. The colour change reagent hydroxynaphthol blue was identified as the best currently available method taking cost, ease of use and reliability into consideration but further work is required to ensure interpretation is consistent between multiple users.

Simple and field-friendly sample preparation was not addressed in this study, but remains an important aspect in the development of LAMP, that has begun to be addressed elsewhere.

The next steps in the development of LAMP as clinical diagnostics for sleeping sickness are Phase 2 evaluation and the development of an integrated protocol including sample preparation, amplification and readout. It remains to be seen whether such a protocol can be developed that is truly operationally feasible, cost-effective and acceptable to end users. The recent collaboration between FIND and Eiken might make this possible. If not, it is reassuring that sleeping sickness diagnostic research is undergoing a small revival, and that LAMP is not the only tool under development. The most notable alternatives are NASBA (another isothermal nucleic acid amplification test) and dipstick style serological tests (being developed by Standard Diagnostics, in collaboration with FIND). In 2009 the number of reported cases of sleeping sickness fell below 10, 000 for the first time in 50 years.

Hopefully, improved diagnosis will assist in the continued fight against this neglected, but deadly disease.

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Appendices

21. LAMP and PCR test-retest agreement using DNA eluted from human blood samples on Whatman FTA cards

22. LAMP and PCR test-retest agreement using pooled data from cattle and human sample sets.

23. LAMP assays with calcein and $MnCl_2$

24. Turbidity verus gel electrophoresis for LAMP *PfrA*, SRALAMP_a and LAMP *TgsGP* for the total set of control DNA samples

25. LAMP for Human African Trypanosomiasis: A Comparative Study of Detection Formats (2010) Sally L. Wastling, Kim Picozzi, Abbas S.L. Kakembo and Susan C. Welburn. PLoS NTDs 4 e865.

Appendix 1. Estimated impact of novel diagnostics for six major developing world diseases

Table 1. Infrastructural requirements, performance characteristics, and impact of novel diagnostics for six major diseases of the developing world [306]

Test for	Requirements of diagnostic		Effect
	Infrastructure	Accuracy (Sensitivity, Specificity)	
Paediatric HIV (<12 months)	Minimal	(90 %, 90 %)	Save 180,000 -2.5 million DALY's over 12 months assuming 5% or 100% population access to ARTs
Paediatic malaria	Minimal	(95 %, 95 %)	Avert 100,000 childhood deaths annually Avert 400,000 unnecessary treatments annually
Paediatric malaria	None	(90 %, 90 %)	Avert 300,000 childhood deaths annually Avert 450 million unnecessary treatments annually

Bacterial acute lower respiratory infections	Minimal	(95 %, 85 %)	Save 400,000 adjusted lives
Diarrhoeal diseases (*Giardia lamblia, Cryptosporidium parvum*, enteroaggregative *Eschericia coli*)	Minimal	(90 %, 90 %)	Save 2.8 million DALY's annually Reduce prevalence of stunted growth by 12.5%
Syphillis	Minimal	(86 %, 72 %)	Save 138 000 adjusted lives, and avert >148 000 still births (assuming 100% treatment rates)
Tuberculosis	None	(85 %, 97 %)	400, 000 adjusted life years annually
Gonorrhoea and Chlamydia	Minimal	(85 %, 90 %)	Save 3 million DALYs Avert 12 million infections Prevent 161 000 related HIV infections in FSW over 4 years

Appendix 2. The sample set of control DNA samples

Table 1. Identity of the 86 samples from which DNA was extracted

No.	Name	Species	Procyclic/Bsf	Origin			Ref
				Host	Date	Country	
1	Tira 24	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
2	Sikuda 28	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
3	UGC	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
4	UGI	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
5	Dal069 IM52	*T. b. gambiense*	Procyclic	Human	1981	Côte d'Ivoire	[246]
6	Papol 33	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
7	Bumanda 146	*T. b. brucei*[a]	Procyclic	Cattle	1988	Uganda	[247]
8	Papol 264	*T. b. rhodesiense*[b]	Bsf	Cattle	1990	Uganda	[247]
9	UGA 88	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
10	E. Oketch	*T. b. rhodesiense*[b]	Procyclic	Human	1990	Uganda	[247]
11	Papol 285	*T. b. rhodesiense*[a]	Procyclic	Cattle	1990	Uganda	[247]
12	Biyamina	*T. b. gambiense*	Bsf	Human	1982	Sudan	[248]
13	Mela 3	*T. b. brucei*[a]	Bsf	Cattle	1988	Uganda	[247]
14	Papol 103	*T. b. rhodesiense*[a]	Bsf	Cattle	1988	Uganda	[247]
15	Mawero 66	*T. b. rhodesiense*[a]	Bsf	Cattle	1990	Uganda	[247]

16	Mela 71 IM87	*T. b. rhodesiense*[a]	Procyclic	Cattle	1998	Uganda	[247]
17	TH149	*T. b. gambiense*	Procyclic	Human	1981	Côte d'Ivoire	[246]
18	Bida 3 Clone A	*T. b. gambiense*	Bsf	Human	1968	Nigeria	[252]
19	TSW83	*T. b. gambiense*	Procyclic	Pig	1981	Côte d'Ivoire	[246]
20	Katerema 311	*T. b. brucei*[a]	Bsf	Cattle	1990	Uganda	[247]
21	Musikia Clone A	*T. b. gambiense*	Bsf	Human	1982	Sudan	[248]
22	Suzena	*T. b. gambiense*	Bsf	Human	1982	Sudan	[248]
23	Tsuaa	*T. b. gambiense*	Bsf	Human	1968	Nigeria	[252]
24	Mawero 32	*T. b. brucei*[c]	Procyclic	Cattle	1990	Uganda	[219]
25	Papol 42	*T. b. brucei*[a]	Procyclic	Cattle	1988	Uganda	[247]
26	UGK	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
27	Papol 60	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
28	UR4	*T. b. brucei*[b]	Procyclic	Fly	1988	Uganda	[247]
29	F97	*T. b. brucei*[b]	Procyclic	Fly	1990	Uganda	[247]
30	Mawero 80	*T. b. brucei*[c]				Uganda	[219]
31	Papol 278	*T. b. rhodesiense*[a]	Procyclic	Cattle	1990	Uganda	[247]
32	URI	*T. b. rhodesiense*[a]	Procyclic	Fly	1988	Uganda	[247]
33	UGH	*T. b. rhodesiense*[a]	Bsf	Human	1988	Uganda	[247]
34	Mela Pig	*T. b. brucei*[a]	Bsf	Pig	1988	Uganda	[247]

35	Tira 22	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
36	Mela 66	*T. b. rhodesiense*[a]	Bsf	Cattle	1988	Uganda	[247]
37	Uganda B	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
38	Mela 24	*T. b. brucei*[a]	Procyclic	Cattle	1990/1988?	Uganda	[247]
39	Iyolowa 125	*T. b. brucei*[a]	Procyclic	Cattle	1988	Uganda	[247]
40	Tira 168	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
41	Papol 12	*T. b. brucei*[a]	Bsf	Cattle	1990	Uganda	[247]
42	Mela 27	*T. b. rhodesiense*[a]	Bsf	Cattle	1988	Uganda	[247]
43	Poyem 11	*T. b. brucei*[a]		Cattle	1988	Uganda	[247]
44	Tira 4	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
45	Mela pig 2	*T. b. brucei*[c]	Bsf	Pig	1990	Uganda	[219]
46	UGE	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
47	Katerema 72		Procyclic	Cattle	1990	Uganda	*
48	Mawero 42	*T. b. rhodesiense*[c]	Procyclic	Cattle	1990	Uganda	[219]
49	Tira 34	*T. congolense*[f]		Cattle	1990	Uganda	*
50	H.Taka	*T. b. rhodesiense*[c]	Procyclic	Human	1990	Uganda	[219]
51	Uganda A	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
52	A Kiburige	*T. b. rhodesiense*[d]	Procyclic	Human	1990	Uganda	*
53	Uganda E	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]

54	Katerema 116	*T. b. rhodesiense*[a]	Procyclic	Cattle	1990	Uganda	[247]
55	Tira 43	*T. b. brucei*[b]	Procyclic	Cattle	1990	Uganda	[247]
56	Tira 92		Procyclic	Cattle	1990	Uganda	*
57	Tira 75	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
58	Musikia Clone B	*T. b. gambiense*	Bsf	Human	1982	Sudan	[248]
59	Iyolowa 116	*T. b. brucei*[a]	Procyclic	Cattle	1988	Uganda	[247]
60	Iyolowa 153	*T. b. brucei*[a]	Procyclic	Cattle	1988	Uganda	[247]
61	TH 112 IM56	*T. b. gambiense*	Procyclic	Human	1981	Côte d'Ivoire	[246]
62	Muraz 15 IM47	*T. b. gambiense*	Procyclic	Human	1980	Burkina Faso	[248]
63	Mela 12	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
64	Mawero 31	*T. b. rhodesiense*[c]	Bsf	Cattle	1990	Uganda	[219]
65	F73	*T. b. rhodesiense*[b]	Procyclic	Fly	1990	Uganda	[247]
66	Tira 27	*T. b. brucei*[a]	Bsf	Cattle	1990	Uganda	[247]
67	Uganda L	*T. b. rhodesiense*[a]	Bsf	Human	1988	Uganda	[247]
68	F48	*T. b. rhodesiense*[b]	Procyclic	Fly	1990	Uganda	[247]
69	Tira 68	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
70	Tira 30	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
71	Tira 29	*T. congolense*[f]	Procyclic	Cattle	1990	Uganda	*
72	Papol 371	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
73	Mawero 85	*T. b. brucei*[c]	Procyclic	Cattle	1990	Uganda	[219]

74	Iyolowa 147	*T. b. brucei*[a]	Bsf	Cattle	1998	Uganda	[247]
75	Muraz 3 IM46	*T. b. gambiense*	Procyclic	Human	1979	Ivory Coast	[248]
76	Katerema 41	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
77	UGG 88	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
78	Mela 2	*T. b. rhodesiense*[a]	Bsf	Cattle	1988	Uganda	[247]
79	Uganda M	*T. b. rhodesiense*[a]	Bsf	Human	1988	Uganda	[247]
80	Rose Akinare	*T. b. rhodesiense*[e]	Procyclic	Human	1990	Uganda	*
81	Uganda B	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
82	Papol 144	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
83	Uganda C	*T. b. rhodesiense*[a]	Procyclic	Human	1988	Uganda	[247]
84	Sikuda 42.90	*T. b. brucei*[a]	Procyclic	Cattle	1990	Uganda	[247]
85	Sikuda 4	*T. congolense*[f]	Procyclic	Cattle		Uganda	*
86	D. Obwang	*T. b. rhodesiense*	Procyclic	Human	1990	Uganda	[184]

a Species identifications made according to human serum resistance *in vitro*, RFLP analysis and if collected from a human host

b Species identification made according to RFLP analysis and if collected from a human host

c Species identified according MGE PCR analysis

d Species identified as *T. b. rhodesiense* according to previously *SRA* positive and collection from a human host (Kim Picozzi, personal communication)

e Assumed to be *T. b. rhodesiense* according to collection from a human host

f Sue Welburn, personal communication

*No reference available but these were collected alongside other samples from the Busoga focus in South East Uganda as described in [247]

Appendix 3. Cost comparison of LAMP *PfrA*, LAMP RIME and TBR PCR

Table 1a. Reagent costs for LAMP *PfrA*

Reagent	**Supplier (catalogue #)**	**Price**	**Quantity for 100 reactions**	**Cost for 100 reactions**
Bst DNA polymerase	New England Biolabs, UK (M0275S)	£49.00 for 1600 units	800 units	£24.50
Thermopol reaction buffer	New England Biolabs, UK	Freely supplied with *Bst* DNA polymerase	250 µl	£0.00
Betaine	Sigma Aldrich, UK (B0300)	£14.80 for 1.5 ml	400 µl	£3.95
dNTPs	Bioline, UK (BIO-39029)	£175.00 for 4 x 500 ml	140 µl	£12.25
$MgSO_4$	New England Biolabs, UK (B9004S)	£10.00 for 1.5 ml supplied with Thermopol reaction buffer	150 µl	£1.00

Water	Sigma Aldrich, UK (W3500)	£6.40 for 100 ml	910 µl	£0.06
Primers	Integrated DNA Technologies	13 p per base		£0.64
Total				**£42.40**

Table 1b. Primer costs for LAMP *PfrA*

Primer	**Number of bases**	**Cost (at 13 p per base)**	**Volume of water added**	**Maximum equivalent volume of 10 µM working stock**	**Amount of working stock required for 100 reactions**	**Cost of primers per 100 reactions**
FIP	42	£5.46	852 µl[a]	7220 µl	400 µl	£0.61
BIP	43	£5.59	722 µl[b]			
F3	19	£2.47	965 µl[c]	9650 µl	50 µl	£0.03
B3	19	£2.47	965 µl			
Total						**£0.64**

a These primers have not been ordered at the 100 nm scale from this company, so this number is taken as an estimate from the LAMP RIME FIP data

b These primers have not been ordered at the 100 nm scale from this company, so this number is taken as an estimate from the LAMP RIME BIP data

c These primers have not been ordered at the 100 nm scale from this company, so this number is taken as an estimate from the LAMP *PfrA* B3 data

Table 2a. Reagent costs for LAMP RIME

Reagent	**Supplier**	**Price**	**Quantity required for 100 reactions**	**Cost for 100 reactions**
Bst DNA polymerase (M0275S)	New England Biolabs, UK	£49.00 for 1600 units	800 units	£24.50
Thermopol reaction buffer (B9004S)	New England Biolabs, UK	Freely supplied with *Bst* DNA polymerase	250 µl	£0.00
Betaine	Sigma Aldrich, UK (B0300)	£14.80 for 1.5 ml	400 µl	£3.95
dNTPs	Bioline,UK (BIO-39029)	£175.00 for 4 x 500 ml	20 µl	£1.75
Water	Sigma Aldrich, UK (W3500)	£6.40 for 100 ml	880 µl	£0.06
Primers	Integrated DNA Technologies	13 p per base		£0.83
Total				**£31.09**

Table 2b. Primer costs for LAMP RIME

Primer	Number of bases	Cost (at 13 p per base)	Volume of water added	Maximum equivalent volume of 10 µM working stock	Amount of working stock required for 100 reactions	Cost of primers per 100 reactions
FIP	42	£5.46	852 µl	7220 µl	500 µl	£0.75
BIP	41	£5.33	722 µl			
LF	18	£2.34	755 µl	5290 µl	200 µl	£0.05
LB	18	£2.34	529 µl			
F3	19	£2.47	764 µl	7640 µl	50 µl	£0.03
B3	20	£2.60	1035 µl			
Total						£0.83

Table 3a. Reagent costs for TBR PCR

Reagent	**Supplier**	**Price**	**Quantity required for 100 reactions**	**Cost for 100 reactions**
BioTaq Red DNA polymerase	Bioline, UK (BIO-21041)	£98.00 for 500 units	100 units	£19.60
NH_4 buffer	Bioline, UK (BIO-21041)	Freely supplied with DNA polymerase	250 µl	£0.00
$MgCl_2$	Bioline, UK (BIO-21041)	Freely supplied with DNA polymerase	75 µl	£0.00
dNTPs	Bioline, UK (BIO-39029)	£175.00 for 4 x 500 ml	20 µl	£1.75
Water	Sigma Aldrich, UK (W3500)	£6.40 for 100 ml	1855 µl	£0.12
Primers	Integrated DNA Technologies	13 p per base		£0.10
Total				**£21.57**

Table 3b. Primer costs for TBR PCR

Primer	Number of bases	Cost (at 13 p per base)	Volume of water added to give a 100 µM stock	Maximum equivalent volume of 10 µM working stock	Amount of working stock required for 100 reactions	Cost of primers per 100 reactions
TBR1	26	£3.38	675 µl	6750 µl	100 µl	**£0.10**
TBR2	24	£3.12	842 µl			

Appendix 4. BLASTing the LAMP RIME primer binding region

Table 1. Fragments of *Trypanozoon* RIME element and *Schistosoma mansoni* showing sequence similarity

***Trypanozoon* gene**	**Gene fragment**	***Schistosoma mansoni* gene**	**Gene fragment**	**% Identity**
EF567424	1-511	FN36744.1	24152-23642	99 %
	7-254		16589-16836	95 %
	258-510		13639-13892	92 %
	258-511		21589-21844	91 %
	15-365	XM_002570283	1-351	99 %
EF567425	1-507	FN36744.1	24151-23642	98 %
	6-252		16589-16835	96 %
	260-507		13645-13892	93 %
	260-507		21595-21844	90 %
	14-361	XM_002570283	1-351	98 %
EF567426	1-512	FN36744.1	24152-23641	99 %
	7-254		16589-16836	95 %
	258-510		13639-13892	92 %
	258-511		21589-21844	91 %
	15-365	XM_002570283	1-351	99 %

Table 2. Binding sites of LAMP RIME primers within *Schistosoma mansoni* FN36744.1

Primer	Binding site
F3	23916-23898
F2	23881-23862
F1c	23841-23820
B1c	23768-23743
B2	23721-23703
B3	23684

Appendix 5. BLASTing the LAMP *PfrA* primer binding region

Table 1. BLAST hits against the *PfrA* gene, X14819, which contain the LAMP *PfrA* primer binding region

Organism	Gene Accession number	% Identity
Trypanosoma cruzi	XM_809076.1	83 %
	FJ222461.1	83 %
	M97548.1	83 %
	AF005195.1	72 %
	AF005194.1	72 %
	AF005193.1	72 %
	XM_804737.1	72 %
Leishmania infantum	AM502234.1	84 %
	XM_001464593.1	80 %
	XM_001464594.1	85 %
Leishmania major	XM_001682174.1	79 %
	XM_001682174.1	79 %
Leishmania mexicana	U45884.1	79 %
Leishmania braziliensis	AM494953.1	79 %
	XM_001563700.1	79 %
Crithidia fasciculata	AY568293.1	79 %

Appendix 6. Novel LAMP primers for *T. b. rhodesiense*

Table 1. SRALAMP primer sets, generated by instructing the primer design software to generate specific primers recognising a mutation at position 581, which span the deletion site in the F1c region. Different colours identify distinct primers.

ID	Deletion recognition region	FIP (F1c-F2) binding site	BIP (B1c-B2) binding site	B3 binding site	F3 binding site
SRALAMP7.1	F1c	(563-583)-(523-544)	(587-611)-(649-666)	(667-688)	(500-517)
SRALAMP7.4	F1c	(563-583)-(523-544)	(588-611)-(649-666)	(667-688)	(500-517)
SRALAMP7.7	F1c	(563-583)-(523-544)	(588-612)-(649-666)	(667-688)	(500-517)
SRALAMP7.10	F1c	(563-583)-(523-544)	(590-614)-(655-674)	(680-697)	(500-517)
SRALAMP7.13	F1c	(563-583)-(523-544)	(604-628)-(655-674)	(680-697)	(500-517)
SRALAMP7.16	F1c	(563-583)-(523-544)	(605-628)-(655-674)	(680-697)	(500-517)

Table 2. SRALAMP primer sets, generated by instructing the primer design software to generate specific primers recognising a mutation at position 581, which span the deletion site in the F2 region. Different colours identify distinct primers

ID	Deletion recognition region	FIP (F1c-F2) binding site	BIP (B1c-B2) binding site	F3 binding site	B3 binding site
SRALAMP7.2	F2	(604-628)-(563-581)	(649-669)-(708-729)	(544-561)	(734-751)
SRALAMP7.3	F2	(605-628)-(565-583)	(649-669)-(708-729)	(545-562)	(747-765)
SRALAMP7.5	F2	(604-628)-(563-581)	(649-670)-(708-729)	(544-561)	(734-751)
SRALAMP7.6	F2	(605-628)-(565-583)	(649-670)-(708-729)	(545-562)	(747-765)
SRALAMP7.8	F2	(604-628)-(563-581)	(651-671)-(708-729)	(544-561)	(734-751)
SRALAMP7.9	F2	(605-628)-(565-583)	(651-671)-(708-729)	(545-562)	(747-765)
SRALAMP7.11	F2	(604-628)-(563-581)	(649-669)-(709-729)	(544-561)	(734-751)
SRALAMP7.12	F2	(605-628)-(565-583)	(649-669)-(709-729)	(545-562)	(747-765)
SRALAMP7.14	F2	(604-628)-(563-581)	(649-670)-(709-729)	(544-561)	(734-751)
SRALAMP7.15	F2	(605-628)-(565-583)	(649-670)-(709-729)	(545-562)	(747-765)
SRALAMP7.17	F2	(604-628)-(563-581)	(651-671)-(709-729)	(544-561)	(734-751)
SRALAMP7.18	F2	(605-628)-	(651-671)-	(545-562)	(747-765)

		(565-583)	(709-729)		

Table 3. Composition of primer sets tested

Primer set	FIP	BIP	F3	B3
SRALAMP7.1	7.1	7.1	7.1	7.1
SRALAMP7.2	7.2	7.2	7.2	7.2
SRALAMP7.3	7.3	7.2	7.3	7.3
SRALAMP7.10	7.1	7.10	7.1	7.10
SRALAMP7.13	7.1	7.13	7.1	7.10

Appendix 7. Binding sites of novel LAMP primers for the specific detection of *T. b. rhodesiense*

Table 1. SRALAMP_a primers

Primer set	Primer	Sequence (5' to 3')	Length
SRALAMP_a	FIP (F1c+F2)	ACGCTATTGGCGCAAGACTT-CATAGTGACAAGATGCGTAC	40
	BIP (B1c+B2)	ACCAGTGGGCACATCTCAGA-TATGCACTTTCCTTCTGTCT	40
	F3	ATCTCAGCGCTTTATGCC	18
	B3	GCCTTATTGCTACTGTTGTT	20
	LF	AGCGTGGACTGCGTTGA	17
	LB	AGTAATCGACATTCTGCAGCAG	22

Figure 1. Binding sites of novel LAMP primers, coded SRALAMP_a, designed using *SRA* (AF097331) and the site of the internal deletion (between nucleotides 579 and 580) in *SRA* when compared with otherwise similar *VSG* (highlighted in red)

AF097331

```
   1 cgctattatt agaacagttt ctgtactata ttgaagacac acctctaaga atcacaatag
  61 caaggcgaac cgaaagcgaa gcacagttaa cgtaacagca atgccccgaa attcgggccg
 121 gacaacaagt accttggcgc tcgcgctggc cctaaagctg ctggcagtgc ctgtatcgcc
 181 cagtggcacc gcctttgacg aagagcccgt caagaaggtt tgcaaagtag aaaaaaactt
 241 agcagacgtc gcaggaatcg ctttggccaa aataaacaac ctgataaaac aagtatcggc
 301 agcaaccgaa gcggaagcaa gaatgacctt ggccgccgca agcacagacc acagcaacat
 361 ctcagcgctt tatgccgcgg cgtcaaacat agtgacaaga tgcgtactca acgcagtcca
```

(F3) (F2)

421 cgctcttaca **agtcttgcgc caatagcgt**t aactgcagcg accaacggag ccaaa**accag**

(F1c) (B1c)

481 **tgggcacatc tcaga**agtaa tcgacattct gcagcaggcg tcacaaggta **agacagaagg**

(B2)

541 **aaagtgcata** gtgaaaagcg gcggcggtac **aacaacagta** **g**caataaggc aactttacaa

(B3)

601 caaaataggg gacctagaaa aacaaacgac caacaactgc ggcaccagcg tgaccgaagt

661 actcgaacac attctaaaac aagaagcgct caaggaagcg ctactttcaa tcgtgaaaaa

721 accaaaaggg gcgccagaca aaacagcagc agatgaattg gtcaccgtgc ttatcaacgg

781 cgtggtgcca aacagcacag cacagaccca aaaattaaag gagaaaattc taaacacctt

841 ggtccccaag cttgtggaag gctcaaaaag ccaagtaaaa ctaaggattc tgaagtaccc

901 gggaaaaata cagaaaagca aactcgtatc aatccaagag ttaaaaaccc gagtggagcc

961 tgaatctagc actgaaagct gcaagcagca ggtcgccacc aaccaggcac aggaggcatt

1021 ttgtaacgca attggcgacg acaaagacaa gggtaacaat gagacacgat gcagttacga

1081 tgacagcaaa ggctcagaca aaaagtgcac atataatgcg gaaaaagcgg aagcaaatgg

1141 ggcacctgca acgcaacctc aaggggagt gaacgaagca acaacaggaa attgtaaagg

1201 gaaactggaa cccggatgca ccaaggcaca agaatacgaa tgggaaggaa aagaatccaa

1261 agattcaagt tttcttgtgg atatgaaatt ggctctgaat atggttgctg cttttgtggc

1321 ctttctgttt taattttcac ctcttttgaa agaactttgc tgtttcatat actttaacac

1381 attttcatga atttgtgaa

Table 2. SRALAMP_b primers

Primer set	Primer	Sequence (5' to 3')	Length
SRALAMP_b	FIP	TTACCTTGTGACGCCTGCTGC-GAGCCAAAACCAGTGGGC	39
	BIP	AACAACAGTAGCAATAAGGC-CGCAGTTGTTGGTCGTTTG	39
	F3	AGTCTTGCGCCAATAGCG	18
	B3	CGAGTACTTCGGTCACGCT	19

Figure 2. Binding sites of novel LAMP primers, coded SRALAMP_b, designed using *SRA* (AF097331) and the site of the internal deletion (between nucleotides 579 and 580) in *SRA* when compared with otherwise similar VSG (highlighted in red)

AF097331

```
   1 cgctattatt agaacagttt ctgtactata ttgaagacac acctctaaga atcacaatag
  61 caaggcgaac cgaaagcgaa gcacagttaa cgtaacagca atgccccgaa attcgggccg
 121 gacaacaagt accttggcgc tcgcgctggc cctaaagctg ctggcagtgc ctgtatcgcc
 181 cagtggcacc gcctttgacg aagagcccgt caagaaggtt tgcaaagtag aaaaaaactt
 241 agcagacgtc gcaggaatcg ctttggccaa aataaacaac ctgataaaac aagtatcggc
 301 agcaaccgaa gcggaagcaa gaatgacctt ggccgccgca agcacagacc acagcaacat
 361 ctcagcgctt tatgccgcgg cgtcaaacat agtgacaaga tgcgtactca acgcagtcca

 421 cgctcttaca agtcttgcgc caatagcgtt aactgcagcg accaacggag ccaaaaccag
                     (F3)                                  (F2)
 481 tgggcacatc tcagaagtaa tcgacattct gcagcaggcg tcacaaggta agacagaagg
                                              (F1c)
 541 aaagtgcata gtgaaaagcg gcggcggtac aacaacagta gcaataaggc aactttacaa
                                           (B1c)
```

```
 601 caaaataggg gacctagaaa aacaaacgac caacaactgc ggcaccagcg tgaccgaagt
                                    (B2)                      (B3)
 661 actcgaacac attctaaaac aagaagcgct caaggaagcg ctactttcaa tcgtgaaaaa

 721 accaaaaggg gcgccagaca aaacagcagc agatgaattg gtcaccgtgc ttatcaacgg
 781 cgtggtgcca aacagcacag cacagaccca aaaattaaag gagaaaattc taaacacctt
 841 ggtccccaag cttgtggaag gctcaaaaag ccaagtaaaa ctaaggattc tgaagtaccc
 901 gggaaaaata cagaaaagca aactcgtatc aatccaagag ttaaaaaccc gagtggagcc
 961 tgaatctagc actgaaagct gcaagcagca ggtcgccacc aaccaggcac aggaggcatt
1021 ttgtaacgca attggcgacg acaaagacaa gggtaacaat gagacacgat gcagttacga
1081 tgacagcaaa ggctcagaca aaaagtgcac atataatgcg gaaaaagcgg aagcaaatgg
1141 ggcacctgca acgcaacctc aaggggagt gaacgaagca acaacaggaa attgtaaagg
1201 gaaactggaa cccggatgca ccaaggcaca agaatacgaa tgggaaggaa aagaatccaa
1261 agattcaagt tttcttgtgg atatgaaatt ggctctgaat atggttgctg cttttgtggc
1321 ctttctgttt taattttcac ctcttttgaa agaactttgc tgtttcatat actttaacac
1381 attttcatga atttgtgaa
```

Table 3. SRALAMP_e primers.

Primer set	Primer	Sequence (5' to 3')	Length
SRALAMP_e	FIP	ACTGGTTTTGGCTCCGTTGG-GTCCACGCTCTTACAAGTC	39
	BIP	GCAGGCGTCACAAGGTAAGA-GCCTTATTGCTACTGTTGTT	40
	F3	GACAAGATGCGTACTCAAC	19
	B3	AGGTCCCCTATTTTGTTGT	19
	LF	CGCTGCAGTTAACGCTATTGG	21
	LB	CAGAAGGAAAGTGCATAGTGAAAAG	25

Figure 3. Binding sites of novel LAMP primers, coded SRALAMP_e, designed using *SRA* (AF097331) and the site of the internal deletion (between nucleotides 579 and 580) in *SRA* when compared with otherwise similar *VSG* (highlighted in red)

AF097331

```
   1 cgctattatt agaacagttt ctgtactata ttgaagacac acctctaaga atcacaatag
  61 caaggcgaac cgaaagcgaa gcacagttaa cgtaacagca atgccccgaa attcgggccg
 121 gacaacaagt accttggcgc tcgcgctggc cctaaagctg ctggcagtgc ctgtatcgcc
 181 cagtggcacc gcctttgacg aagagcccgt caagaaggtt tgcaaagtag aaaaaaactt
 241 agcagacgtc gcaggaatcg ctttggccaa aataaacaac ctgataaaac aagtatcggc
 301 agcaaccgaa gcggaagcaa gaatgacctt ggccgccgca agcacagacc acagcaacat
 361 ctcagcgctt tatgccgcgg cgtcaaacat agtgacaaga tgcgtactca acgcagtcca
                                          (F3)              (F2)
 421 cgctcttaca agtcttgcgc caatagcgtt aactgcagcg accaacggag ccaaaaccag
                                               (F1c)
 481 tgggcacatc tcagaagtaa tcgacattct gcagcaggcg tcacaaggta agacagaagg
                                           (B1c)
 541 aaagtgcata gtgaaaagcg gcggcggtac aacaacagta gcaataaggc aactttacaa
                                        (B2)                    (B3)
 601 caaaataggg gacctagaaa aacaaacgac caacaactgc ggcaccagcg tgaccgaagt

 661 actcgaacac attctaaaac aagaagcgct caaggaagcg ctactttcaa tcgtgaaaaa
 721 accaaaaggg gcgccagaca aaacagcagc agatgaattg gtcaccgtgc ttatcaacgg
 781 cgtggtgcca aacagcacag cacagaccca aaaattaaag gagaaaattc taaacacctt
 841 ggtccccaag cttgtggaag gctcaaaaag ccaagtaaaa ctaaggattc tgaagtaccc
 901 gggaaaaata cagaaaagca aactcgtatc aatccaagag ttaaaaaccc gagtggagcc
 961 tgaatctagc actgaaagct gcaagcagca ggtcgccacc aaccaggcac aggaggcatt
1021 ttgtaacgca attggcgacg acaaagacaa gggtaacaat gagacacgat gcagttacga
```

```
1081 tgacagcaaa ggctcagaca aaaagtgcac atataatgcg gaaaaagcgg aagcaaatgg
1141 ggcacctgca acgcaacctc aaggggggagt gaacgaagca acaacaggaa attgtaaagg
1201 gaaactggaa cccggatgca ccaaggcaca agaatacgaa tgggaaggaa aagaatccaa
1261 agattcaagt tttcttgtgg atatgaaatt ggctctgaat atggttgctg cttttgtggc
1321 ctttctgttt taattttcac ctcttttgaa agaactttgc tgtttcatat actttaacac
1381 attttcatga atttgtgaa
```

Table 4. SRALAMP_f primers

Primer set	Primer	Sequence (5' to 3')	Length
SRALAMP_f	FIP	GCCTTATTGCTACTGTTGTT-CAGGCGTCACAAGGTAAG	38
	BIP	AATAGGGGACCTAGAAAAACAAACG-GAATGTGTTCGAGTACTTCG	45
	F3	GTGGGCACATCTCAGAAG	18
	B3	AAGTAGCGCTTCCTTGAG	18
	LF	GCTTTTCACTATGCACTTTCCTTC	24
	LB	ACAACTGCGGCACCAGC	27

Figure 4. Binding sites of novel LAMP primers, coded SRALAMP_f, designed using *SRA* (AF097331) and the site of the internal deletion (between nucleotides 579 and 580) in *SRA* when compared with otherwise similar *VSG* (highlighted in red)

```
AF097331
   1 cgctattatt agaacagttt ctgtactata ttgaagacac acctctaaga atcacaatag
  61 caaggcgaac cgaaagcgaa gcacagttaa cgtaacagca atgccccgaa attcgggccg
 121 gacaacaagt accttggcgc tcgcgctggc cctaaagctg ctggcagtgc ctgtatcgcc
 181 cagtggcacc gcctttgacg aagagcccgt caagaaggtt tgcaaagtag aaaaaaactt
```

241 agcagacgtc gcaggaatcg ctttggccaa aataaacaac ctgataaaac aagtatcggc

301 agcaaccgaa gcggaagcaa gaatgacctt ggccgccgca agcacagacc acagcaacat

361 ctcagcgctt tatgccgcgg cgtcaaacat agtgacaaga tgcgtactca acgcagtcca

421 cgctcttaca agtcttgcgc caatagcgtt aactgcagcg accaacggag ccaaaacca**g**

481 **tgggcacatc tcagaag**taa tcgacattct gcag**caggcg tcacaaggta ag**acagaagg

(F3) (F2)

541 aaagtgcata gtgaaaagcg gcggcggtac **aacaacagta gcaataaggc** aactttacaa

(F1c)

601 caa**ataggg gacctagaaa aacaaacg**ac caacaactgc ggcaccagcg tgac**cgaagt**

(B1c) (F2)

661 **actcgaacac attc**taaaac aagaagcg**ct caaggaagcg ctactt**tcaa tcgtgaaaaa

(B3)

721 accaaaaggg gcgccagaca aaacagcagc agatgaattg gtcaccgtgc ttatcaacgg

781 cgtggtgcca aacagcacag cacagaccca aaaattaaag gagaaaattc taaacacctt

841 ggtccccaag cttgtggaag gctcaaaaag ccaagtaaaa ctaaggattc tgaagtaccc

901 gggaaaaata cagaaaagca aactcgtatc aatccaagag ttaaaaaccc gagtggagcc

961 tgaatctagc actgaaagct gcaagcagca ggtcgccacc aaccaggcac aggaggcatt

1021 ttgtaacgca attggcgacg acaaagacaa gggtaacaat gagacacgat gcagttacga

1081 tgacagcaaa ggctcagaca aaaagtgcac atataatgcg gaaaaagcgg aagcaaatgg

1141 ggcacctgca acgcaacctc aaggggggagt gaacgaagca acaacaggaa attgtaaagg

1201 gaaactggaa cccggatgca ccaaggcaca agaatacgaa tgggaaggaa aagaatccaa

1261 agattcaagt tttcttgtgg atatgaaatt ggctctgaat atggttgctg cttttgtggc

1321 ctttctgttt taattttcac ctcttttgaa agaactttgc tgtttcatat actttaacac

1381 attttcatga atttgtgaa

Table 5. SRALAMP7.1 primers

Primer set	Primer	Sequence (5' to 3')	Length
SRALAMP7.1	FIP (F1c+F2)	TGCTACTGTTGTTGTACCGCC-ACAAGGTAAGACAGAAGGAAAG	43
	BIP (B1c+B2)	AGGCAACTTTACAACAAAATAGGGG-TCGAGTACTTCGGTCACG	43
	F3	ATCGACATTCTGCAGCAG	18
	B3	CGCTTCTTGTTTTAGAATGTGT	22

Figure 5. Binding sites of novel LAMP primers, coded SRALAMP7.1, designed using *SRA* (AF097331) and the site of the internal deletion (between nucleotides 579 and 580) in *SRA* when compared with otherwise similar *VSG* (highlighted in red)

AF097331

```
   1 cgctattatt agaacagttt ctgtactata ttgaagacac acctctaaga atcacaatag
  61 caaggcgaac cgaaagcgaa gcacagttaa cgtaacagca atgccccgaa attcgggccg
 121 gacaacaagt accttggcgc tcgcgctggc cctaaagctg ctggcagtgc ctgtatcgcc
 181 cagtggcacc gcctttgacg aagagcccgt caagaaggtt tgcaaagtag aaaaaaactt
 241 agcagacgtc gcaggaatcg ctttggccaa aataaacaac ctgataaaac aagtatcggc
 301 agcaaccgaa gcggaagcaa gaatgacctt ggccgccgca agcacagacc acagcaacat
 361 ctcagcgctt tatgccgcgg cgtcaaacat agtgacaaga tgcgtactca acgcagtcca

 421 cgctcttaca agtcttgcgc caatagcgtt aactgcagcg accaacggag ccaaaaccag

 481 tgggcacatc tcagaagtaa tcgacattct gcagcaggcg tcacaaggta agacagaagg
                                (F3)                    (F2)
 541 aaagtgcata gtgaaaagcg gcggcggtac aacaacagta gcaataaggc aactttacaa
                                        (F1c)                (B1c)
```

601 **caaaataggg g**acctagaaa aacaaacgac caacaactgc ggcaccag**cg tgaccgaagt**

(B2)

661 **actcga acac attctaaaac aagaagcg**ct caaggaagcg ctactttcaa tcgtgaaaaa

(B3)

721 accaaaaggg gcgccagaca aaacagcagc agatgaattg gtcaccgtgc ttatcaacgg

781 cgtggtgcca aacagcacag cacagaccca aaaattaaag gagaaaattc taaacacctt

841 ggtccccaag cttgtggaag gctcaaaaag ccaagtaaaa ctaaggattc tgaagtaccc

901 gggaaaaata cagaaaagca aactcgtatc aatccaagag ttaaaaaccc gagtggagcc

961 tgaatctagc actgaaagct gcaagcagca ggtcgccacc aaccaggcac aggaggcatt

1021 ttgtaacgca attggcgacg acaaagacaa gggtaacaat gagacacgat gcagttacga

1081 tgacagcaaa ggctcagaca aaaagtgcac atataatgcg gaaaaagcgg aagcaaatgg

1141 ggcacctgca acgcaacctc aaggggagt gaacgaagca acaacaggaa attgtaaagg

1201 gaaactggaa cccggatgca ccaaggcaca agaatacgaa tgggaaggaa aagaatccaa

1261 agattcaagt tttcttgtgg atatgaaatt ggctctgaat atggttgctg cttttgtggc

1321 ctttctgttt taattttcac ctcttttgaa agaactttgc tgtttcatat actttaacac

1381 attttcatga atttgtgaa

Table 6. SRALAMP7.2 Primers

Primer set	Primer	Sequence (5’ to 3’)	Length
SRALAMP7.2	FIP	CGTTTGTTTTTCTAGGTCCCCTATTGG CGGTACAACAACAGTAG	44
	BIP	CGTGACCGAAGTACTCGAACACCTTT TGGTTTTTTCACGATTG	43
	F3	GTGCATAGTGAAAAGCGG	18
	B3	TGCTGCTGTTTTGTCTGG	18

Figure 6. Binding sites of novel LAMP primers, coded SRALAMP7.2, designed using *SRA* (AF097331) and the site of the internal deletion (between nucleotides 579 and 580) in *SRA* when compared with otherwise similar *VSG* (highlighted in red)

AF097331

```
   1 cgctattatt agaacagttt ctgtactata ttgaagacac acctctaaga atcacaatag
  61 caaggcgaac cgaaagcgaa gcacagttaa cgtaacagca atgccccgaa attcgggccg
 121 gacaacaagt accttggcgc tcgcgctggc cctaaagctg ctggcagtgc ctgtatcgcc
 181 cagtggcacc gcctttgacg aagagcccgt caagaaggtt tgcaaagtag aaaaaaactt
 241 agcagacgtc gcaggaatcg ctttggccaa aataaacaac ctgataaaac aagtatcggc
 301 agcaaccgaa gcggaagcaa gaatgacctt ggccgccgca agcacagacc acagcaacat
 361 ctcagcgctt tatgccgcgg cgtcaaacat agtgacaaga tgcgtactca acgcagtcca

 421 cgctcttaca agtcttgcgc caatagcgtt aactgcagcg accaacggag ccaaaaccag

 481 tgggcacatc tcagaagtaa tcgacattct gcagcaggcg tcacaaggta agacagaagg

 541 aaagtgcata gtgaaaagcg gcggcggtac aacaacagta gcaataaggc aactttacaa
                (F3)             (F2)
 601 caaaataggg gacctagaaa aacaaacgac caacaactgc ggcaccagcg tgaccgaagt
            (F1c)                                          (B1c)
 661 actcgaacac attctaaaac aagaagcgct caaggaagcg ctactttcaa tcgtgaaaaa
                                                      (B2)
 721 accaaaaggg gcgccagaca aaacagcagc agatgaattg gtcaccgtgc ttatcaacgg
                       (B3)
 781 cgtggtgcca aacagcacag cacagaccca aaaattaaag gagaaaattc taaacacctt
 841 ggtccccaag cttgtggaag gctcaaaaag ccaagtaaaa ctaaggattc tgaagtaccc
 901 gggaaaaata cagaaaagca aactcgtatc aatccaagag ttaaaaaccc gagtggagcc
 961 tgaatctagc actgaaagct gcaagcagca ggtcgccacc aaccaggcac aggaggcatt
1021 ttgtaacgca attggcgacg acaaagacaa gggtaacaat gagacacgat gcagttacga
```

```
1081 tgacagcaaa ggctcagaca aaaagtgcac atataatgcg gaaaaagcgg aagcaaatgg
1141 ggcacctgca acgcaacctc aaggggggagt gaacgaagca acaacaggaa attgtaaagg
1201 gaaactggaa cccggatgca ccaaggcaca agaatacgaa tgggaaggaa aagaatccaa
1261 agattcaagt tttcttgtgg atatgaaatt ggctctgaat atggttgctg cttttgtggc
1321 ctttctgttt taattttcac ctcttttgaa agaactttgc tgtttcatat actttaacac
1381 attttcatga atttgtgaa
```

Table 7. SRALAMP7.3 primers

Primer set	Primer	Sequence (5' to 3')	Length
SRALAMP7.3	FIP (F1c+F2)	CGTTTGTTTTTCTAGGTCCCCTATCGG TACAACAACAGTAGCA	43
	BIP (B1c+B2)	CGTGACCGAAGTACTCGAACACCTTT TGGTTTTTTCACGATTG	43
	F3	TGCATAGTGAAAAGCGGC	18
	B3	GTGACCAATTCATCTGCTG	19

Figure 7. Binding sites of novel LAMP primers, coded SRALAMP7.3 , designed using *SRA* (AF097331) and the site of the internal deletion (between nucleotides 579 and 580) in SRA when compared with otherwise similar VSG (highlighted in red)

```
AF097331
   1 cgctattatt agaacagttt ctgtactata ttgaagacac acctctaaga atcacaatag
  61 caaggcgaac cgaaagcgaa gcacagttaa cgtaacagca atgccccgaa attcgggccg
 121 gacaacaagt accttggcgc tcgcgctggc cctaaagctg ctggcagtgc ctgtatcgcc
 181 cagtggcacc gcctttgacg aagagcccgt caagaaggtt tgcaaagtag aaaaaaactt
 241 agcagacgtc gcaggaatcg ctttggccaa aataaacaac ctgataaaac aagtatcggc
 301 agcaaccgaa gcggaagcaa gaatgacctt ggccgccgca agcacagacc acagcaacat
```

361 ctcagcgctt tatgccgcgg cgtcaaacat agtgacaaga tgcgtactca acgcagtcca

421 cgctcttaca agtcttgcgc caatagcgtt aactgcagcg accaacggag ccaaaaccag

481 tgggcacatc tcagaagtaa tcgacattct gcagcaggcg tcacaaggta agacagaagg

541 aaag**tgcata gtgaaaagcg gc**gg**cggtac aacaacagt**a **gc**aataaggc aactttacaa

(F3) (F2)

601 caaa**ataggg gacctagaaa aacaaacg**ac caacaactgc ggcaccag**cg tgaccgaagt**

(F1c) (B1c)

661 **actcgaaca**c attctaaaac aagaagcgct caaggaagcg ctactt**caa tcgtgaaaaa**

(B2)

721 **accaaaagg**g gcgccagaca aaacag**cagc agatgaattg gtcac**cgtgc ttatcaacgg

(B3)

781 cgtggtgcca aacagcacag cacagaccca aaaattaaag gagaaaattc taaacacctt

841 ggtccccaag cttgtggaag gctcaaaaag ccaagtaaaa ctaaggattc tgaagtaccc

901 gggaaaaata cagaaaagca aactcgtatc aatccaagag ttaaaaaccc gagtggagcc

961 tgaatctagc actgaaagct gcaagcagca ggtcgccacc aaccaggcac aggaggcatt

1021 ttgtaacgca attggcgacg acaaagacaa gggtaacaat gagacacgat gcagttacga

1081 tgacagcaaa ggctcagaca aaaagtgcac atataatgcg gaaaaagcgg aagcaaatgg

1141 ggcacctgca acgcaacctc aaggggggagt gaacgaagca acaacaggaa attgtaaagg

1201 gaaactggaa cccggatgca ccaaggcaca agaatacgaa tgggaaggaa aagaatccaa

1261 agattcaagt tttcttgtgg atatgaaatt ggctctgaat atggttgctg cttttgtggc

1321 ctttctgttt taattttcac ctcttttgaa agaactttgc tgtttcatat actttaacac

1381 attttcatga atttgtgaa

Table 8. SRALAMP7.10 primers

Primer set	Primer	Sequence (5' to 3')	Length
SRALAMP7.10	FIP (F1c+F2)	TGCTACTGTTGTTGTACCGCC-ACAAGGTAAGACAGAAGGAAAG	43
	BIP (B1c+B2)	CAACTTTACAACAAAATAGGGGACC GAATGTGTTCGAGTACTTCG	45
	F3	ATCGACATTCTGCAGCAG	18
	B3	CAACTTTACAACAAAATAGGGGACC GAATGTGTTCGAGTACTTCG	18

Figure 8. Binding sites of novel LAMP primers, coded SRALAMP7.10, designed using *SRA* (AF097331) and the site of the internal deletion (between nucleotides 579 and 580) in *SRA* when compared with otherwise similar *VSG* (highlighted in red)

AF097331

```
   1 cgctattatt agaacagttt ctgtactata ttgaagacac acctctaaga atcacaatag
  61 caaggcgaac cgaaagcgaa gcacagttaa cgtaacagca atgccccgaa attcgggccg
 121 gacaacaagt accttggcgc tcgcgctggc cctaaagctg ctggcagtgc ctgtatcgcc
 181 cagtggcacc gcctttgacg aagagcccgt caagaaggtt tgcaaagtag aaaaaaactt
 241 agcagacgtc gcaggaatcg ctttggccaa aataaacaac ctgataaaac aagtatcggc
 301 agcaaccgaa gcggaagcaa gaatgacctt ggccgccgca agcacagacc acagcaacat
 361 ctcagcgctt tatgccgcgg cgtcaaacat agtgacaaga tgcgtactca acgcagtcca

 421 cgctcttaca agtcttgcgc caatagcgtt aactgcagcg accaacggag ccaaaaccag

 481 tgggcacatc tcagaagta a tcgacattct gcagcag gcg tc acaaggta agacagaagg
                                (F3)                    (F2)
```

```
 541 aaagtgcata gtgaaaagcg gcggcggtac aacaacagta gcaataaggc aactttacaa
                                        (F1c)                (B1c)
 601 caaaataggg gacctagaaa aacaaacgac caacaactgc ggcaccagcg tgaccgaagt
                                                                (B2)
 661 actcgaacac attctaaaac aagaagcgct caaggaagcg ctactttcaa tcgtgaaaaa
                                (B3)
 721 accaaaaggg gcgccagaca aaacagcagc agatgaattg gtcaccgtgc ttatcaacgg
 781 cgtggtgcca aacagcacag cacagaccca aaaattaaag gagaaaattc taaacacctt
 841 ggtccccaag cttgtggaag gctcaaaaag ccaagtaaaa ctaaggattc tgaagtaccc
 901 gggaaaaata cagaaaagca aactcgtatc aatccaagag ttaaaaaccc gagtggagcc
 961 tgaatctagc actgaaagct gcaagcagca ggtcgccacc aaccaggcac aggaggcatt
1021 ttgtaacgca attggcgacg acaaagacaa gggtaacaat gagacacgat gcagttacga
1081 tgacagcaaa ggctcagaca aaaagtgcac atataatgcg gaaaaagcgg aagcaaatgg
1141 ggcacctgca acgcaacctc aaggggagt gaacgaagca acaacaggaa attgtaaagg
1201 gaaactggaa cccggatgca ccaaggcaca agaatacgaa tgggaaggaa aagaatccaa
1261 agattcaagt tttcttgtgg atatgaaatt ggctctgaat atggttgctg cttttgtggc
1321 ctttctgttt taattttcac ctcttttgaa agaactttgc tgtttcatat actttaacac
1381 attttcatga atttgtgaa
```

Table 9. SRALAMP7.13 primers

Primer set	Primer	Sequence (5' to 3')	Length
SRALAMP7.13	FIP (F1c+F2)	TGCTACTGTTGTTGTACCGCC-ACAAGGTAAGACAGAAGGAAAG	43
	BIP (B1c+B2)	AATAGGGGACCTAGAAAAACAAACG GAATGTGTTCGAGTACTTCG	45
	F3	ATCGACATTCTGCAGCAG	18
	B3	TTCCTTGAGCGCTTCTTG	18

Figure 9. Binding sites of novel LAMP primers, coded SRALAMP7.13, designed using *SRA* (AF097331) and the site of the internal deletion (between nucleotides 579 and 580) in *SRA* when compared with otherwise similar *VSG* (highlighted in red)

AF097331

```
   1 cgctattatt agaacagttt ctgtactata ttgaagacac acctctaaga atcacaatag
  61 caaggcgaac cgaaagcgaa gcacagttaa cgtaacagca atgccccgaa attcgggccg
 121 gacaacaagt accttggcgc tcgcgctggc cctaaagctg ctggcagtgc ctgtatcgcc
 181 cagtggcacc gcctttgacg aagagcccgt caagaaggtt tgcaaagtag aaaaaaactt
 241 agcagacgtc gcaggaatcg ctttggccaa aataaacaac ctgataaaac aagtatcggc
 301 agcaaccgaa gcggaagcaa gaatgacctt ggccgccgca agcacagacc acagcaacat
 361 ctcagcgctt tatgccgcgg cgtcaaacat agtgacaaga tgcgtactca acgcagtcca

 421 cgctcttaca agtcttgcgc caatagcgtt aactgcagcg accaacggag ccaaaaccag

 481 tgggcacatc tcagaagtaa tcgacattct gcagcaggcg tcacaaggta agacagaagg
                                  (F3)                    (F2)
 541 aaagtgcata gtgaaaagcg gcggcggtac aacaacagta gcaataaggc aactttacaa
                                        (F1c)
```

601 caa**aataggg gacctagaaa aacaaacg**ac caacaactgc ggcaccagcg tgac**cgaagt**

(B1c) (B2)

661 **actcgaacac attc**taaaa**c aagaagcgct caaggaa**gcg ctactttcaa tcgtgaaaaa

(B3)

721 accaaaaggg gcgccagaca aaacagcagc agatgaattg gtcaccgtgc ttatcaacgg

781 cgtggtgcca aacagcacag cacagaccca aaaattaaag gagaaaattc taaacacctt

841 ggtccccaag cttgtggaag gctcaaaaag ccaagtaaaa ctaaggattc tgaagtaccc

901 gggaaaaata cagaaaagca aactcgtatc aatccaagag ttaaaaaccc gagtggagcc

961 tgaatctagc actgaaagct gcaagcagca ggtcgccacc aaccaggcac aggaggcatt

1021 ttgtaacgca attggcgacg acaaagacaa gggtaacaat gagacacgat gcagttacga

1081 tgacagcaaa ggctcagaca aaaagtgcac atataatgcg gaaaaagcgg aagcaaatgg

1141 ggcacctgca acgcaacctc aagggggagt gaacgaagca acaacaggaa attgtaaagg

1201 gaaactggaa cccggatgca ccaaggcaca agaatacgaa tgggaaggaa aagaatccaa

1261 agattcaagt tttcttgtgg atatgaaatt ggctctgaat atggttgctg cttttgtggc

1321 ctttctgttt taattttcac ctcttttgaa agaactttgc tgtttcatat actttaacac

1381 attttcatga atttgtgaa

Appendix 8. Results from the *Trypanozoon* and *T. b. rhodesiense* PCR and LAMP assays

Sample	[DNA] (ng µl^{-1})	TBR PCR	LAMP *PfrA* (Tt)	*SRA* PCR			LAMP *SRA*1
				VSG	*SRA*	*GPI-PLC*	
1. Tira 24	6.48	+	00:37:24	+	-	+	-
2. Sikuda 28	47.25	+	00:37:36	+	-	+	-
3. UGC	0.01	+	00:35:12	+	+	+	+
4. UGI	1.01	+	00:36:36	+	+	+	+
5. Dal069 IM52	73.49	+	00:36:48	+	+	+	-
6. Papol 33	21.96	+	00:35:54	+	-	+	-
7. Bumanda 146	60.14	+	00:36:00	+	-	+	-
8. Papol 264	69.57	+	00:36:24	+	-	+	-
9. UGA 88	22.53	+	00:45:36	+	+	+	+
10. E. Oketch	15.21	+	00:50:06	+	+	+	+
11. Papol 285	12.12	+	00:48:18	+	+	+	+
12. Biyamina	26.87	+	00:47:54	+	+	+	+
13. Mela 3	24.09	+	00:48:54	+	-	+	-
14. Papol 103	5.77	+	00:51:00	+	+	+	+
15. Mawero 66	18.37	+	00:49:18	+	+	+	+
16. Mela 71 IM87	23.3	+	00:48:06	+	+	+	+
17. TH149	20.31	+	00:42:42	+	-	+	-
18. Bida 3 Clone A	53.14	-	00:46:12	+	-	+	-

19. TSW83	27.45	+	00:40:06	+	-	+	-
20. Katerema 311	7.39	+	00:46:00	+	-	+	-
21. Musikia Clone A	5.72	-	00:52:24	-	-	+	-
22. Suzena	8.13	+	00:52:48	-	-	+	-
23. Tsuaa	14.77	+	00:49:00	-	-	+	-
24. Mawero 32	4.62	+	00:43:36	+	-	+	-
25. Papol 42	1.7	+	00:34:36	+	-	+	-
26. UGK	9.57	+	00:33:12	+	+	+	+
27. Papol 60	0.32	+	00:31:54	+	-	+	-
28. UR4	49.11	+	00:32:36	+	-	+	-
29. F97	2.75	+	00:32:12	+	-	+	-
30. Mawero 80	5.49	+	00:33:24	+	-	+	-
31. Papol 278	7.64	+	00:42:00	+	+	+	+
32. URI	18.15	+	00:40:54	+	+	+	+
33. UGH	1.87	+	00:29:18	+	+	+	+
34. Mela Pig	7.19	+	00:30:36	+	-	+	-
35. Tira 22	4.44	+	00:29:00	+	-	+	-
36. Mela 66	6.01	+	00:29:24	+	+	+	+
37. Uganda B	8.66	+	00:28:00	+	+	+	+
38. Mela 24	4.34	+	00:28:42	+	-	+	-
39. Iyolowa 125	13.15	+	00:28:54	+	-	+	-
40. Tira 168	4.98	+	00:27:42	+	-	+	+
41. Papol 12	1.45	+	00:31:12	+	-	+	-

42. Mela 27	5	+	00:31:30	+	+	+	+
43. Poyem 11	10.6	+	00:31:36	+	-	+	-
44. Tira 4	5.13	+	00:31:06	+	-	+	-
45. Mela pig 2	17.89	+	00:30:18	+	-	+	-
46. UGE	6.05	+	00:28:48	+	+	+	+
47. Katerema 72	1.92	+	00:31:54	+	+	+	-
48. Mawero 42	7.92	+	00:30:36	+	+	+	+
49. Tira 34	1.71	+	00:38:18	+	-	+	-
50. H.Taka	20.26	+	00:29:42	+	+	+	+
51. Uganda A	9.04	+	00:30:30	+	+	+	+
52. A Kiburige	26.28	+	00:31:54	+	+	+	+
53. Uganda E	6.04	+	00:30:42	+	+	+	+
54. Katerema 116	14.12	+	00:30:24	+	+	+	-
55. Tira 43	5.81	+	00:30:42	+	-	+	-
56. Tira 92	13.14	+	00:00:00	-	-	-	-
57. Tira 75	4.93	+	00:36:30	+	-	+	-
58. Musikia Clone B	9.11	+	00:30:00	-	-	+	-
59. Iyolowa 116	3.94	+	00:25:48	+	-	+	-
60. Iyolowa 153	5.36	+	00:27:48	+	-	+	-
61. TH 112 IM56	21.32	+	00:29:00	+	-	+	-
62. Muraz 15 IM47	24.87	+	00:00:00	+	-	+	-
63. Mela 12	6.78	+	00:35:48	+	-	+	-

64. Mawero 31	24.32	+	00:33:54	+	+	+	+
65. F73	7.7	+	00:49:42	+	+	+	+
66. Tira 27	30.69	+	00:53:54	+	-	+	-
67. Uganda L	3.19	+	00:46:54	+	+	+	+
68. F48	8.76	+	00:47:12	+	+	+	+
69. Tira 68	11.96	+	00:47:30	+	-	+	-
70. Tira 30	1.52	+	00:48:00	+	-	+	-
71. Tira 29	1.55	-	00:00:00	+	-	+	-
72. Papol 371	144.78	+	00:00:00	+	-	+	-
73. Mawero 85	6.83	+	00:49:00	+	-	+	-
74. Iyolowa 147	1.33	+	00:45:54	+	-	+	-
75. Muraz 3 IM46	29.59	+	00:46:00	+	-	+	-
76. Katerema 41	5.51	+	00:47:24	+	-	+	-
77. UGG 88	24.29	+	00:47:54	+	+	+	+
78. Mela 2	3.73	+	00:49:06	+	+	+	+
79. Uganda M	2.17	+	00:52:06	+	+	+	+
80. Rose Akinare	2.39	+	00:48:18	+	+	+	+
81. Uganda B	0.79	+	00:44:54	+	+	+	+
82. Papol 144	2.88	+	00:46:12	+	-	+	-
83. Uganda C	9.76	+	00:47:00	+	+	+	+
84. Sikuda 42.90	8.99	+	00:44:42	+	-	+	-
85. Sikuda 4	6.3	-	00:00:00	-	-	-	-
86. D. Obwang	8.2	+	00:41:12	+	+	+	+

Appendix 9. Results from the SRALAMP_a assay applied to the total sample set

Sample	TBR PCR	LAMP *PfrA* (Tt)	*SRA* PCR	SRALAMP_a by gel			SRALAMP_a by turbidity			SRALAMP_a (Tt)
				i	ii	iii	i	ii	iii	
1. Tira 24	+	00:37:24	-	-	+	-	-	+	-	00:00:00
2. Sikuda 28	+	00:37:36	-	-	-	-	-	-	-	00:42:48
3. UGC	+	00:35:12	+	+	+	+	+	+	+	00:34:06
4. UGI	+	00:36:36	+	+	+	+	+	+	+	00:35:30
5. Dal069 IM52	+	00:36:48	+	-	-	-	-	-	-	00:00:00
6. Papol 33	+	00:35:54	-	-	-	-	-	-	-	00:00:00
7. Bumanda 146	+	00:36:00	-	-	-	-	-	-	-	00:00:00
8. Papol 264	+	00:36:24	-	-	-	-	-	-	-	00:00:00
9. UGA 88	+	00:45:36	+	+	+	+	+	+	+	00:33:24
10. E. Oketch	+	00:50:06	+	+	+	+	-	+	+	00:35:54
11. Papol 285	+	00:48:18	+	+	+	+	-	+	+	00:35:06
12. Biyamina	+	00:47:54	+	+	+	+	-	+	+	00:36:30

13. Mela 3	+	00:48:54	-	-	-	-	-	-	-	00:00:00
14. Papol 103	+	00:51:00	+	+	+	+	+	+	+	00:37:24
15. Mawero 66	+	00:49:18	+	+	+	+	-	+	+	00:40:42
16. Mela 71 IM87	+	00:48:06	+	+	+	+	-	+	+	00:34:54
17. TH149	+	00:42:42	-	-	-	-	-	-	-	00:00:00
18. Bida 3 Clone A	-	00:46:12	-	-	+	+	-	-	+	00:00:00
19. TSW83	+	00:40:06	-	-	-	-	-	-	-	00:00:00
20. Katerema 311	+	00:46:00	-	-	-	-	-	-	-	00:00:00
21. Musikia Clone A	-	00:52:24	-	-	-	-	-	-	-	00:45:24
22. Suzena	+	00:52:48	-	-	-	-	-	-	-	00:00:00
23. Tsuaa	+	00:49:00	-	-	-	-	-	-	-	01:01:06
24. Mawero 32	+	00:43:36	-	-	-	+	-	-	+	00:39:06
25. Papol 42	+	00:34:36	-	-	-	-	-	-	-	00:00:00
26. UGK	+	00:33:12	+	+	+	+	-	+	-	00:41:36

27. Papol 60	+	00:31:54	-	-	-	-	-	-	-	00:00:00
28. UR4	+	00:32:36	-	-	-	-	-	-	-	00:00:00
29. F97	+	00:32:12	-	-	-	-	-	-	-	00:00:00
30. Mawero 80	+	00:33:24	-	+	-	-	-	-	-	00:00:00
31. Papol 278	+	00:42:00	+	+	+	+	-	+	-	00:48:54
32. URI	+	00:40:54	+	+	+	+	-	+	-	00:48:18
33. UGH	+	00:29:18	+	+	+	+	-	+	+	00:53:00
34. Mela Pig	+	00:30:36	-	-	-	-	-	-	-	00:00:00
35. Tira 22	+	00:29:00	-	-	-	-	-	-	-	00:00:00
36. Mela 66	+	00:29:24	+	+	+	+	-	+	+	00:29:48
37. Uganda B	+	00:28:00	+	+	+	+	-	+	+	00:43:54
38. Mela 24	+	00:28:42	-	-	-	-	-	-	-	00:00:00
39. Iyolowa 125	+	00:28:54	-	-	-	+	-	-	+	00:00:00
40. Tira 168	+	00:27:42	-	-	-	+	-	-	+	00:00:00
41. Papol 12	+	00:31:12	-	-	-	+	-	-	+	00:00:00
42. Mela	+	00:31:30	+	+	+	+	-	+	+	00:51:18

27										
43. Poyem 11	+	00:31:36	-	-	-	+	-	-	+	00:00:00
44. Tira 4	+	00:31:06	-	-	-	-	-	-	-	00:00:00
45. Mela pig 2	+	00:30:18	-	-	-	+	-	-	+	00:00:00
46. UGE	+	00:28:48	+	+	+	+	+	+	+	00:31:24
47. Katerema 72	+	00:31:54	+	-	-	-	-	-	-	00:40:36
48. Mawero 42	+	00:30:36	+	+	+	+	-	+	+	00:33:36
49. Tira 34	+	00:38:18	-	-	-	+	-	-	+	00:00:00
50. H.Taka	+	00:29:42	+	+	+	+	-	+	+	00:33:00
51. Uganda A	+	00:30:30	+	+	+	+	-	+	+	00:34:00
52. A Kiburige	+	00:31:54	+	+	+	+	-	+	+	00:32:54
53. Uganda E	+	00:30:42	+	+	+	+	-	+	+	00:33:18
54. Katerema 116	+	00:30:24	+	+	+	+	-	+	+	00:33:12
55. Tira 43	+	00:30:42	-	-	-	+	-	-	+	00:45:48

56. Tira 92	+	00:00:00	Insufficient DNA	-	-	-	-	-	-	00:00:00
57. Tira 75	+	00:36:30	-	+	-	-	-	-	-	00:00:00
58. Musikia Clone B	+	00:30:00	-	-	-	-	-	-	-	00:00:00
59. Iyolowa 116	+	00:25:48	-	-	+	-	-	+	-	00:00:00
60. Iyolowa 153	+	00:27:48	-	-	-	-	-	-	-	00:00:00
61. TH 112 IM56	+	00:29:00	-	-	-	-	-	-	-	00:00:00
62. Muraz 15 IM47	+	00:00:00	-	-	-	-	-	-	-	00:00:00
63. Mela 12	+	00:35:48	-	-	+	-	-	-	-	00:00:00
64. Mawero 31	+	00:33:54	+	+	+	+	+	+	+	00:38:06
65. F73	+	00:49:42	+	+	+	+	+	+	+	00:33:54
66. Tira 27	+	00:53:54	-	-	-	-	-	-	-	00:00:00
67. Uganda L	+	00:46:54	+	+	+	+	+	+	+	00:31:48
68. F48	+	00:47:12	+	+	+	+	+	+	+	00:33:24

69. Tira 68	+	00:47:30	-	-	-	+	-	-	+	00:00:00
70. Tira 30	+	00:48:00	-	-	-	-	-	-	-	00:00:00
71. Tira 29	-	00:00:00	-	-	+	-	-	+	-	00:00:00
72. Papol 371	+	00:00:00	-	-	-	-	-	-	-	00:00:00
73. Mawero 85	+	00:49:00	-	-	-	-	-	-	-	00:00:00
74. Iyolowa 147	+	00:45:54	-	-	-	-	-	-	-	00:00:00
75. Muraz 3 IM46	+	00:46:00	-	-	-	-	-	-	-	00:00:00
76. Katerema 41	+	00:47:24	-	-	-	-	-	-	-	00:53:18
77. UGG 88	+	00:47:54	+	+	+	+	+	+	+	00:40:06
78. Mela 2	+	00:49:06	+	+	+	+	+	+	+	00:41:24
79. Uganda M	+	00:52:06	+	+	+	+	+	+	+	00:44:48
80. Rose Akinare	+	00:48:18	+	+	+	+	+	+	+	00:41:54
81. Uganda B	+	00:44:54	+	+	+	+	+	+	+	00:40:30

82. Papol 144	+	00:46:12	-	-	-	-	-	-	-	00:00:00
83. Uganda C	+	00:47:00	+	+	+	+	+	+	+	00:39:18
84. Sikuda 42.90	+	00:44:42	-	-	-	+	-	-	+	00:00:00
85. Sikuda 4	-	00:00:00	Insufficient DNA	-	-	-	-	-	-	00:54:18

Appendix 10. Primer binding sites for the published LAMP *SRA*1 and novel SRALAMP_a primers in relation to the internal deletion site which distinguishes *SRA*

(The internal deletion site is highlighted in bold red type)

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SeqA Name                          Len(nt)  SeqB Name                          Len(nt)  Score
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1    gi|19913120|emb|AJ345058.1|   1190     2    gi|19913118|emb|AJ345057.1|   1190     98
1    gi|19913120|emb|AJ345058.1|   1190     3    gi|24940358|emb|Z37159.2|     1398     97
1    gi|19913120|emb|AJ345058.1|   1190     4    gi|3851625|gb|AF097331.1|     1399     97
2    gi|19913118|emb|AJ345057.1|   1190     3    gi|24940358|emb|Z37159.2|     1398     99
2    gi|19913118|emb|AJ345057.1|   1190     4    gi|3851625|gb|AF097331.1|     1399     99
3    gi|24940358|emb|Z37159.2|     1398     4    gi|3851625|gb|AF097331.1|     1399     99
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CLUSTAL 2.0.12 multiple sequence alignment


gi|19913118|emb|AJ345057.1|      --------------------------------------------------
gi|24940358|emb|Z37159.2|        CGCTATTATTAGAACAGTTTCTGTACTATATTGA---CACATCTCTAAGA 47
gi|3851625|gb|AF097331.1|        CGCTATTATTAGAACAGTTTCTGTACTATATTGAAGACACACCTCTAAGA 50
gi|19913120|emb|AJ345058.1|      --------------------------------------------------


gi|19913118|emb|AJ345057.1|      --------------------------------------------------
gi|24940358|emb|Z37159.2|        ATCACAATAGCAAGGCGAACCGAAAGCGAAGCACAGTTAACGTAACAGCA 97
gi|3851625|gb|AF097331.1|        ATCACAATAGCAAGGCGAACCGAAAGCGAAGCACAGTTAACGTAACAGCA 100
gi|19913120|emb|AJ345058.1|      --------------------------------------------------


gi|19913118|emb|AJ345057.1|      --------------------GACAACAAGTACCTTGGCGCTCGCGCTGGC 30
gi|24940358|emb|Z37159.2|        ATGCCCCGAAATTCGGGCCGGACAACAAGTACCTTGGCGCTCGCCGTGGC 147
gi|3851625|gb|AF097331.1|        ATGCCCCGAAATTCGGGCCGGACAACAAGTACCTTGGCGCTCGCGCTGGC 150
gi|19913120|emb|AJ345058.1|      --------------------GACAACAAGTACCTTGGCGCTCGCGCTGGC 30
                                                    ************************  ****


gi|19913118|emb|AJ345057.1|      CCTAAAGCTGCTGGCAGTGCCTGTATCGCCCAGTGGCACCGCCTTTGACG 80
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gi|24940358|emb|Z37159.2|       CCTAAAGCTGCTGGCAGTGCCTGTATCGCCCAGTGGCACCGCCTTTGACG 197
gi|3851625|gb|AF097331.1|       CCTAAAGCTGCTGGCAGTGCCTGTATCGCCCAGTGGCACCGCCTTTGACG 200
gi|19913120|emb|AJ345058.1|     CCTAAAGCTGCTGGCAGTGCCTGTATCGCCCAGTGGCCCCGCCTTTGACG 80
                                ************************************ ************

gi|19913118|emb|AJ345057.1|     AAGAGCCCGTCAAGAAGGTTTGCAAAGTAGAAAAAAACTTAGCAGACGTC 130
gi|24940358|emb|Z37159.2|       AAGAGCCCGTCAAGAAGGTTTGCAAAGTAGAAAAAAACTTAGCAGACGTC 247
gi|3851625|gb|AF097331.1|       AAGAGCCCGTCAAGAAGGTTTGCAAAGTAGAAAAAAACTTAGCAGACGTC 250
gi|19913120|emb|AJ345058.1|     AAGAGCCCGTCAAGAAGGTTTGCAAAGTAGAAAAAAACTTAGCAGACGTC 130
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gi|19913118|emb|AJ345057.1|     GCAGGAATCGCTTTGGCCAAAATAAACAACCTGATAAAACAAGTATCGGC 180
gi|24940358|emb|Z37159.2|       GCAGGAATCGCTTTGGCCAAAATAAACAACCTGATAAAACAAGTATCGGC 297
gi|3851625|gb|AF097331.1|       GCAGGAATCGCTTTGGCCAAAATAAACAACCTGATAAAACAAGTATCGGC 300
gi|19913120|emb|AJ345058.1|     GCAGGAATCGCTTTGGCCAAAATAAACAACCTGATAAAACAAGTATCGGC 180
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gi|19913118|emb|AJ345057.1|     AGCAACCGAAGCGGAAGCAAGAATGACCTTGGCCGCCGCAAGCACAGACC 230
gi|24940358|emb|Z37159.2|       AGCAACCGAAGCGGAAGCAAGAATGACCTTGGCCGCCGCAAGCACAGACC 347
gi|3851625|gb|AF097331.1|       AGCAACCGAAGCGGAAGCAAGAATGACCTTGGCCGCCGCAAGCACAGACC 350
gi|19913120|emb|AJ345058.1|     AGCAACCGAAGCGGAAGCAAGACTGACCTTGGCCGCTGCAAGCACAGACC 230
                                ********************** ************* *************
                                          < LAMP SRA1 F3   >        < LAMP SRA1 F2

gi|19913118|emb|AJ345057.1|     ACAGCAACATCTCAGCGCTTTATGCCGCGGCGTCAAACATAGTGACAAGA 280
gi|24940358|emb|Z37159.2|       ACAGCAACATCTCAGCGCTTTATGCCGCGGCGTCAAACATAGTGACAAGA 397
gi|3851625|gb|AF097331.1|       ACAGCAACATCTCAGCGCTTTATGCCGCGGCGTCAAACATAGTGACAAGA 400
gi|19913120|emb|AJ345058.1|     ACAGCAACATCTCAGCGCTTTATGCCGCGGCGTCAAACATAGTGACAAGA 280
                                *************************************************
                                    >     <  LAMP SRA1 LF   >                  <

gi|19913118|emb|AJ345057.1|     TGCGTACTCAACGCAGTCCACGCTCTTACAAGTCTTGCGCCAATAGCGTT 330
gi|24940358|emb|Z37159.2|       TGCGTACTCAACGCAGTCCACGCTCTTACAAGTCTTGCGCCAATAGCGTT 447
gi|3851625|gb|AF097331.1|       TGCGTACTCAACGCAGTCCACGCTCTTACAAGTCTTGCGCCAATAGCGTT 450
gi|19913120|emb|AJ345058.1|     TGCGTACTCAACGCAGTCCACGCTCTTACAAGTCTTGCGCCAATAGCGTT 330
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                                  LAMP SRA1 F1c   > <   LAMP SRA1 B1c   >

gi|19913118|emb|AJ345057.1|     AACTGCAGCGACCAACGGAGCCAAAACCAGTGGGCACATCTCAGAAGTAA 380
gi|24940358|emb|Z37159.2|       AACTGCAGCGACCAACGGAGCCAAAACCAGTGGGCACATCTCAGAAGTAA 497
gi|3851625|gb|AF097331.1|       AACTGCAGCGACCAACGGAGCCAAAACCAGTGGGCACATCTCAGAAGTAA 500
gi|19913120|emb|AJ345058.1|     AACTGCAGCGACCAACGGAGCCAAAACCAGTGGGCACATCTCAGAAGTAA 380
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                                    <  LAMP SRA1 LB  >      <   LAMP SRA1 B2   >

gi|19913118|emb|AJ345057.1|      TCGACATTCTGCAGCAGGCGTCACAAGGTAAGACAGAAGGAAAGTGCATA 430
gi|24940358|emb|Z37159.2|        TCGACATTCTGCAGCAGGCGTCACAAGGTAAGACAGAAGGAAAGTGCATA 547
gi|3851625|gb|AF097331.1|        TCGACATTCTGCAGCAGGCGTCACAAGGTAAGACAGAAGGAAAGTGCATA 550
gi|19913120|emb|AJ345058.1|      TCGACATTCTGCAGCAGGCGTCACAAGGTAAGACAGAAGGAAAGTGCATA 430
                                 *********************************************
                                                <  LAMP SRA1 B3   >

gi|19913118|emb|AJ345057.1|      GTGAAAAGCGGCGGCGGTACAACAACAGTAGCAATAAGGCAACTTTACAA 480
gi|24940358|emb|Z37159.2|        GTGAAAAGCGGCGGCGGTACAACAACAGTAGCAATAAGGCAACTTTACAA 597
gi|3851625|gb|AF097331.1|        GTGAAAAGCGGCGGCGGTACAACAACAGTAGCAATAAGGCAACTTTACAA 600
gi|19913120|emb|AJ345058.1|      GTGAAAAGCGGCGGCGGTACAACAACAGTAGCAATAAGGCAACTTTACAA 480
                                 *********************************************

gi|19913118|emb|AJ345057.1|      CAAAATAGGGGACCTAGAAAAACAAACGACCAACAACTGCGGCACCAGCG 530
gi|24940358|emb|Z37159.2|        CAAAATAGGGGACCTAGAAAAACAAACGACCAACAACTGCGGCACCAGCG 647
gi|3851625|gb|AF097331.1|        CAAAATAGGGGACCTAGAAAAACAAACGACCAACAACTGCGGCACCAGCG 650
gi|19913120|emb|AJ345058.1|      CAAAATAGGGGACCTAGAAAAACAAACGACCAACAACTGCGGCACCAGCG 530
                                 *********************************************

gi|19913118|emb|AJ345057.1|      TGACCGAAGTACTCGAACACATTCTAAAACAAGAAGCGCTCAAGGAAGCG 580
gi|24940358|emb|Z37159.2|        TGACCGAAGTACTCGAACACATTCTAAAACAAGAAGCGCTCAAGGAAGCC 697
gi|3851625|gb|AF097331.1|        TGACCGAAGTACTCGAACACATTCTAAAACAAGAAGCGCTCAAGGAAGCG 700
gi|19913120|emb|AJ345058.1|      TGACCGAAGTACTCGAACACATTCTAAAACAAGAAGCGCTCAAGGAAGCG 580
                                 *********************************************

gi|19913118|emb|AJ345057.1|      CTACTTTCAATCGTGAAAAAACCAAAAGGGGCGCCAGACAAAACAGCAGC 630
gi|24940358|emb|Z37159.2|        GTACTTTCAATCGTGAAAAAACCAAAAGGGGCGCCAGACAAAACAGCAGC 747
gi|3851625|gb|AF097331.1|        CTACTTTCAATCGTGAAAAAACCAAAAGGGGCGCCAGACAAAACAGCAGC 750
gi|19913120|emb|AJ345058.1|      CTACTTTCAATCGTGAAAAAACCAAAAGGGGCGCCAGACAAAACAGCAGC 630
                                  ********************************************

gi|19913118|emb|AJ345057.1|      AGATGAATTGGTCACCGCGCTAATCAACGGCGTGGTGCCAAACAGCACAG 680
gi|24940358|emb|Z37159.2|        AGATGAATTGGTCACCGCGCTAATCAACGGCGTGGTGCCAAACAGCACAG 797
gi|3851625|gb|AF097331.1|        AGATGAATTGGTCACCGTGCTTATCAACGGCGTGGTGCCAAACAGCACAG 800
gi|19913120|emb|AJ345058.1|      AGATGAAATGGTCACCGCGCTAATCAACGGCGTGGTGCCAAACAGCACAG 680
                                 ******* ********* *** ****************************

gi|19913118|emb|AJ345057.1|      CACAGACCCAAAAATTAAAGGAGAAAATTCTAAACACCTTGGTCCCCAAG 730
gi|24940358|emb|Z37159.2|        CACAGACCCAAAAATTAAAGGAGAAAATTCTAAACACCTTGGTCCCCAAG 847
gi|3851625|gb|AF097331.1|        CACAGACCCAAAAATTAAAGGAGAAAATTCTAAACACCTTGGTCCCCAAG 850
gi|19913120|emb|AJ345058.1|      CACAGACCCAAAAATTAAAGGAGAAAATTCTAAACACCTTGGTCCCCAAG 730
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gi|19913118|emb|AJ345057.1|       CTTGTGGAAGGCTCAAAAAGCCAAGTAAAACTAAGGATTCTGAAGTACCC 780
gi|24940358|emb|Z37159.2|         CTTGTGGAAGGCTCAAAAAGCCAAGTAAAACTAAGGATTCTGAAGTACCC 897
gi|3851625|gb|AF097331.1|         CTTGTGGAAGGCTCAAAAAGCCAAGTAAAACTAAGGATTCTGAAGTACCC 900
gi|19913120|emb|AJ345058.1|       CTTGTGGAAGGCTCAAAAAGCCAAGTAAAACTAAGGATTCTGAAGTACCC 780
                                  ************************************************

gi|19913118|emb|AJ345057.1|       GGGAAAAATACAGAAAAGCAAACTCGTATCAATCCAAGAGTTAAAAACCC 830
gi|24940358|emb|Z37159.2|         GGGAAAAATACAGAAAAGCAAACTCGTATCAATCCAAGAGTTAAAAACCC 947
gi|3851625|gb|AF097331.1|         GGGAAAAATACAGAAAAGCAAACTCGTATCAATCCAAGAGTTAAAAACCC 950
gi|19913120|emb|AJ345058.1|       GGGAAAAATACAGAAAAGCAAACTCGTATCAATCCAAGAGTTAAAAACCC 830
                                  ************************************************

gi|19913118|emb|AJ345057.1|       GAGTGGAGCCTGAATCTAGCACTGAAAGCTGCAAGCAGCAGGTCGCCACC 880
gi|24940358|emb|Z37159.2|         GAGTGGAGCCTGAATCTAGCACTGAAAGCTGCAAGCAGCAGGTCGCCACC 997
gi|3851625|gb|AF097331.1|         GAGTGGAGCCTGAATCTAGCACTGAAAGCTGCAAGCAGCAGGTCGCCACC 1000
gi|19913120|emb|AJ345058.1|       GAGTGGAGCCTGAATCTAGCACTGAAAGCTGCAAGCAGCAGGTCGCCACC 880
                                  ************************************************

gi|19913118|emb|AJ345057.1|       AACCAGGCACAGGAGGCATTTTGTAACGCAATTGGCGACGACAAAGACAA 930
gi|24940358|emb|Z37159.2|         AACCAGGCACAGGAGGCATTTTGTAACGCAATTGGCGACGACAAAGACAA 1047
gi|3851625|gb|AF097331.1|         AACCAGGCACAGGAGGCATTTTGTAACGCAATTGGCGACGACAAAGACAA 1050
gi|19913120|emb|AJ345058.1|       AACCAGGCACAGGAGGCATTTTGTAACGCAATTGGCGACGACAAAGACAA 930
                                  ************************************************

gi|19913118|emb|AJ345057.1|       GTGTAACAATGAGACACGATGCAGTTACGATGACAGCAAAGGCTCAGACA 980
gi|24940358|emb|Z37159.2|         GTGTAACAATGAGACACGATGCAGTTACGATGACAGCAAAGGCTCAGACA 1097
gi|3851625|gb|AF097331.1|         GGGTAACAATGAGACACGATGCAGTTACGATGACAGCAAAGGCTCAGACA 1100
gi|19913120|emb|AJ345058.1|       GTGTAACAATGAGAAACGATGCAGTTACGATGACAGCAAAGGCTCAGGAA 980
                                  * ************ ********************************  *

gi|19913118|emb|AJ345057.1|       AAAAGTGCACATATAATGCGGAAAAAGCAGAAGCAAATGGGGCACCTGCA 1030
gi|24940358|emb|Z37159.2|         AAAAGTGCACATATAATGCGGAAAAAGCAGAAGCAAATGGGGCACCTGCA 1147
gi|3851625|gb|AF097331.1|         AAAAGTGCACATATAATGCGGAAAAAGCGGAAGCAAATGGGGCACCTGCA 1150
gi|19913120|emb|AJ345058.1|       AAAAGTGCACATATAATGCCACAAAAGCCGCAGAAAATGGAGTTCCTGCA 1030
                                  ******************   ****** * ** ****** *  ******

gi|19913118|emb|AJ345057.1|       ACGCAACCTCAAGGGGGAGTGAACGAAGCAACAACAGGAAATTGTAAAGG 1080
gi|24940358|emb|Z37159.2|         ACGCAACCTCAAGGGGGAGTGAACGAAGCAACAACAGGAAATTGTAAAGG 1197
gi|3851625|gb|AF097331.1|         ACGCAACCTCAAGGGGGAGTGAACGAAGCAACAACAGGAAATTGTAAAGG 1200
gi|19913120|emb|AJ345058.1|       ACGCAACATCAAACGGGAGGGACTGAAGCAACAACAGGAAATTGTAAAGG 1080
                                  ******* ****  ***** **  *************************
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gi|19913118|emb|AJ345057.1|      GAAACTGGAACCCGGATGCACCAAGGCACAAGAATACGAATGGGAAGGAA 1130
gi|24940358|emb|Z37159.2|        GAAACTGGAACCCGGATGCACCAAGGCACAAGAATACGAATGGGAAGGAA 1247
gi|3851625|gb|AF097331.1|        GAAACTGGAACCCGGATGCACCAAGGCACAAGAATACGAATGGGAAGGAA 1250
gi|19913120|emb|AJ345058.1|      GAAACTGGAACCCGGATGCACCAAGGCACAAGAATACGAATGGGAAGGAA 1130
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gi|19913118|emb|AJ345057.1|      AAGAATCCAAAGATTCAAGTTTTCTTGTGGATATGAAATTGGCTCTGAAT 1180
gi|24940358|emb|Z37159.2|        AAGAATCCAAAGATTCAAGTTTTCTTGTGGATATGAAATTGGCTCTGAAT 1297
gi|3851625|gb|AF097331.1|        AAGAATCCAAAGATTCAAGTTTTCTTGTGGATATGAAATTGGCTCTGAAT 1300
gi|19913120|emb|AJ345058.1|      AAGAATCCAAAGATTCAAGTTTTCTTGTGGATATGAAATTGGCTCTGAAT 1180
                                 **************************************************

gi|19913118|emb|AJ345057.1|      ATGGTTGCTG---------------------------------------- 1190
gi|24940358|emb|Z37159.2|        ATGGTTGCTGCTTTTGTGGCCTTTCTGTTTTAATTTTCACCTCTTTTGAA 1347
gi|3851625|gb|AF097331.1|        ATGGTTGCTGCTTTTGTGGCCTTTCTGTTTTAATTTTCACCTCTTTTGAA 1350
gi|19913120|emb|AJ345058.1|      ATGGTTGCTG---------------------------------------- 1190
                                 **********

gi|19913118|emb|AJ345057.1|      --------------------------------------------------
gi|24940358|emb|Z37159.2|        AGAACTTTGCTGTTTCATATACTTTAACACATTTTCATGAATTTGTGAAA 1397
gi|3851625|gb|AF097331.1|        AGAACTTTGCTGTTTCATATACTTTAACACATTTTCATGAATTTGTGAA- 1399
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gi|3851625|gb|AF097331.1|        -
gi|19913120|emb|AJ345058.1|      -
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SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
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1	gi|19913120|emb|AJ345058.1|	1190	3	gi|24940358|emb|Z37159.2|	1398	97
1	gi|19913120|emb|AJ345058.1|	1190	4	gi|3851625|gb|AF097331.1|	1399	97
2	gi|19913118|emb|AJ345057.1|	1190	3	gi|24940358|emb|Z37159.2|	1398	99
2	gi|19913118|emb|AJ345057.1|	1190	4	gi|3851625|gb|AF097331.1|	1399	99

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3    gi|24940358|emb|Z37159.2|     1398     4    gi|3851625|gb|AF097331.1|     1399     99
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CLUSTAL 2.0.12 multiple sequence alignment


gi|19913118|emb|AJ345057.1|      --------------------------------------------------
gi|24940358|emb|Z37159.2|        CGCTATTATTAGAACAGTTTCTGTACTATATTGA---CACATCTCTAAGA 47
gi|3851625|gb|AF097331.1|        CGCTATTATTAGAACAGTTTCTGTACTATATTGAAGACACACCTCTAAGA 50
gi|19913120|emb|AJ345058.1|      --------------------------------------------------


gi|19913118|emb|AJ345057.1|      --------------------------------------------------
gi|24940358|emb|Z37159.2|        ATCACAATAGCAAGGCGAACCGAAAGCGAAGCACAGTTAACGTAACAGCA 97
gi|3851625|gb|AF097331.1|        ATCACAATAGCAAGGCGAACCGAAAGCGAAGCACAGTTAACGTAACAGCA 100
gi|19913120|emb|AJ345058.1|      --------------------------------------------------


gi|19913118|emb|AJ345057.1|      --------------------GACAACAAGTACCTTGGCGCTCGCGCTGGC 30
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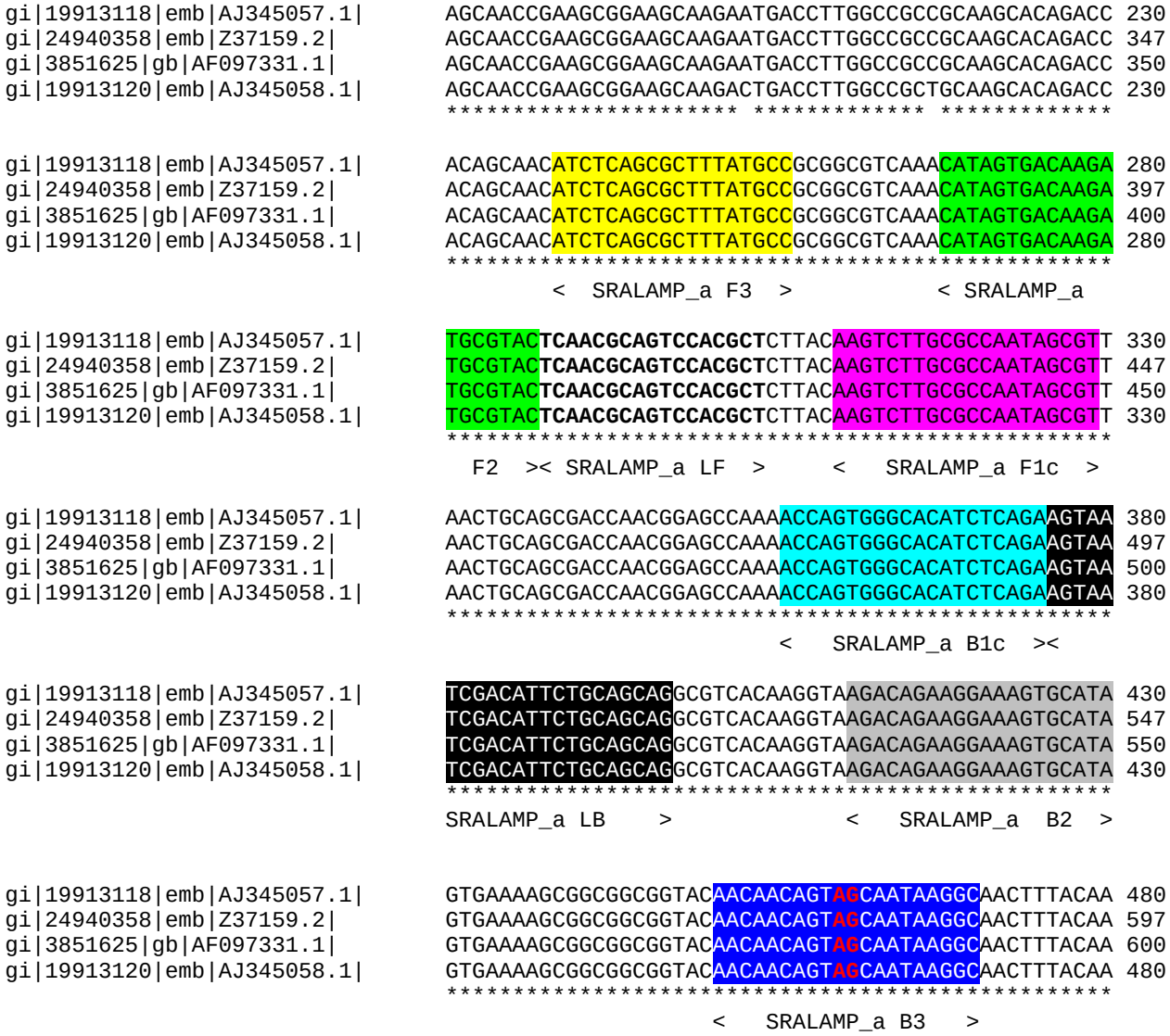
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gi|3851625|gb|AF097331.1|        CAAAATAGGGGACCTAGAAAAACAAACGACCAACAACTGCGGCACCAGCG 650
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Appendix 11. Previous isoenzyme analyses of *T. b. gambiense* stocks used in this study

(Group 2 *T. b. gambiense* highlighted in bold)

From Godfrey *et al.* 1987 [248]

Sample	Virulence to rodents	ALAT	ASAT	ICD	PEP1	PEP2	PGM	ME	NH	SOD
12. Biyamina	Low	1	2	2	13	9	2	2	1	3
21. Musikia Cl A	Low	1	2	2	13	9	2	2	1	3
22. Suzena	Low	1	2	2	13	9	2	2	1	3
58. Musikia Cl B	Low	1	2	2	13	9	2	2	1	3
62. Muraz 15	Low	1	2	2	1	6	2	2	3	Not done
75. Muraz 3	**High**	**2**	**1**	**2**	**2**	**1**	**6**	**1**	**1**	**Not done**

From Mehlitz *et al.* 1982 [246]

Sample	ALAT	ASAT	ICD	PEP1	PEP2	PGM	ME	Zymodeme
5. Dal069	I	II	II	I	VI	II	II	A
17. TH149	I	III	II	I	VI	II	II	D
19. TSW 83	**II**	**I**	**II**	**I**	**I**	**II**	**XV**	**C**
61. TH112	**X**	**I**	**II**	**II**	**I**	**II**	**I**	**I**

From Godfrey and Kilgour, 1976 [252]

Sample	ALAT	ASAT
18. Bida 3	I	II
23. Tsuaa	I	I

Appendix 12 Results from the *Trypanozoon* and *T. b. gambiense* PCR and LAMP assays.

Sample	[DNA] (ng µl^{-1})	PCR		LAMP *PfrA* (Tt)	LAMP TBG1	
		TBR	*TgsGP*		Tt	Gel
1. Tira 24	6.48	+	-	00:37:24	01:22:00	+
2. Sikuda 28	47.25	+	-	00:37:36	01:23:48	+
3. UGC	0.01	+	-	00:35:12	01:12:06	+
4. UGI	1.01	+	-	00:36:36	01:07:12	+
5. Dal069 IM52	73.49	+	+	00:36:48	01:08:30	+
6. Papol 33	21.96	+	-	00:35:54	01:07:42	+
7. Bumanda 146	60.14	+	-	00:36:00	01:11:12	+
8. Papol 264	69.57	+	-	00:36:24	01:09:06	+
9. UGA 88	22.53	+	-	00:45:36	01:01:42	+
10. E. Oketch	15.21	+	-	00:50:06	01:13:42	+
11. Papol 285	12.12	+	-	00:48:18	01:19:24	+
12. Biyamina	26.87	+	-	00:47:54	01:13:36	-
13. Mela 3	24.09	+	-	00:48:54	01:23:30	+
14. Papol 103	5.77	+	-	00:51:00	01:15:42	+
15. Mawero 66	18.37	+	-	00:49:18	01:17:24	+
16. Mela 71 IM87	23.3	+	-	00:48:06	01:07:48	+
17. TH149	20.31	+	+	00:42:42	01:10:18	-

18. Bida 3 Clone A	53.14	-	+	00:46:12	01:17:42	-
19. TSW83	27.45	+	-	00:40:06	00:54:24	-
20. Katerema 311	7.39	+	+	00:46:00	00:00:00	+
21. Musikia Clone A	5.72	-	+	00:52:24	00:00:00	-
22. Suzena	8.13	+	+	00:52:48	00:00:00	-
23. Tsuaa	14.77	+	+	00:49:00	01:13:48	+
24. Mawero 32	4.62	+	-	00:43:36	01:10:18	+
25. Papol 42	1.7	+	-	00:34:36	01:25:30	+
26. UGK	9.57	+	-	00:33:12	01:25:24	+
27. Papol 60	0.32	+	-	00:31:54	01:10:54	+
28. UR4	49.11	+	-	00:32:36	01:18:30	+
29. F97	2.75	+	-	00:32:12	01:05:12	+
30. Mawero 80	5.49	+	-	00:33:24	01:11:42	-
31. Papol 278	7.64	+	-	00:42:00	01:12:48	-
32. URI	18.15	+	-	00:40:54	01:05:54	+
33. UGH	1.87	+	+	00:29:18	01:17:06	+
34. Mela Pig	7.19	+	-	00:30:36	00:00:00	-
35. Tira 22	4.44	+	-	00:29:00	01:12:00	-
36. Mela 66	6.01	+	-	00:29:24	01:20:42	-
37. Uganda B	8.66	+	-	00:28:00	01:17:00	+
38. Mela 24	4.34	+	-	00:28:42	01:12:54	-

39. Iyolowa 125	13.15	+	-	00:28:54	01:17:54	+
40. Tira 168	4.98	+	-	00:27:42	01:14:30	-
41. Papol 12	1.45	+	-	00:31:12	01:18:12	-
42. Mela 27	5	+	-	00:31:30	01:15:54	+
43. Poyem 11	10.6	+	-	00:31:36	01:19:54	+
44. Tira 4	5.13	+	-	00:31:06	01:12:30	-
45. Mela pig 2	17.89	+	-	00:30:18	01:07:30	+
46. UGE	6.05	+	-	00:28:48	01:08:42	+
47. Katerema 72	1.92	+	-	00:31:54	01:22:42	+
48. Mawero 42	7.92	+	-	00:30:36	01:13:06	-
49. Tira 34	1.71	+	-	00:38:18	00:00:00	-
50. H.Taka	20.26	+	-	00:29:42	01:09:54	-
51. Uganda A	9.04	+	-	00:30:30	01:19:54	-
52. A Kiburige	26.28	+	-	00:31:54	01:13:12	-
53. Uganda E	6.04	+	-	00:30:42	01:08:06	-
54. Katerema 116	14.12	+	-	00:30:24	01:25:06	+
55. Tira 43	5.81	+	-	00:30:42	01:22:54	-
56. Tira 92	13.14	+	-	00:00:00	00:00:00	-
57. Tira 75	4.93	+	-	00:36:30	01:24:12	+
58. Musikia Clone B	9.11	+	+	00:30:00	01:28:12	-
59. Iyolowa 116	3.94	+	-	00:25:48	00:00:00	-

60. Iyolowa 153	5.36	+	-	00:27:48	01:27:12	-
61. TH 112 IM56	21.32	+	-	00:29:00	01:17:48	+
62. Muraz 15 IM47	24.87	+	-	00:00:00	01:16:12	-
63. Mela 12	6.78	+	-	00:35:48	01:22:42	-
64. Mawero 31	24.32	+	-	00:33:54	01:19:00	+
65. F73	7.7	+	-	00:49:42	00:00:00	+
66. Tira 27	30.69	+	-	00:53:54	00:00:00	-
67. Uganda L	3.19	+	-	00:46:54	01:19:54	+
68. F48	8.76	+	-	00:47:12	01:18:42	+
69. Tira 68	11.96	+	-	00:47:30	01:25:06	-
70. Tira 30	1.52	+	-	00:48:00	00:00:00	-
71. Tira 29	1.55	-	-	00:00:00	00:00:00	-
72. Papol 371	144.78	+	-	00:00:00	00:00:00	-
73. Mawero 85	6.83	+	-	00:49:00	00:00:00	+
74. Iyolowa 147	1.33	+	-	00:45:54	00:00:00	-
75. Muraz 3 IM46	29.59	+	-	00:46:00	01:27:48	+
76. Katerema 41	5.51	+	-	00:47:24	00:00:00	+
77. UGG 88	24.29	+	-	00:47:54	00:00:00	+
78. Mela 2	3.73	+	-	00:49:06	01:09:30	-
79. Uganda M	2.17	+	-	00:52:06	01:28:12	-
80. Rose Akinare	2.39	+	-	00:48:18	01:11:48	-

81. Uganda B	0.79	+	-	00:44:54	01:11:12	+
82. Papol 144	2.88	+	-	00:46:12	01:15:24	+
83. Uganda C	9.76	+	-	00:47:00	01:14:54	-
84. Sikuda 42.90	8.99	+	-	00:44:42	01:23:00	+
85. Sikuda 4	6.3	-	-	00:00:00	00:00:00	-
86. D. Obwang	8.2	+	-	00:41:12	01:12:00	+

Appendix 13. Primer binding sites for LAMP TBG1 primers.

Table 1. Primer binding site for LAMP TBG1 primers

Primer	Binding site
F3	AAGCTCTCTCGAGCCATC
F2	ATGCCACAT TTCTCAGTGT
F1c	CACCTATTTTGTGTTGTTCAACGC
B1c	CCACCTCT TCTCCTCGTGTG
B2	TACGATATCTTTCATCTCTTTCTTC
B3	TCGCACATCTTGTGTATGTCA

Figure 1. LAMP TBG1 primer binding sites for four *T. b. brucei* and four *T. b. gambiense* ITS1-5.8S rRNA-ITS2 sequences from Agbo *et al.* [249]

Key: The C_3A insertion in *T. b. gambiense* is highlighted in capital letters. The primer binding sites are indicated in colour according to Table 1 above. Differences between primer and sequence are underlined.

***T. b. brucei* H3, AF306770**

```
  1 tggtgcaata caggtgatcg gaccgtcgct cgtctcgggc gaccgaagtt caccgatatt
 61 gcttcaatag aggaagcaaa agtcgtaaca aggtagctgt aggtgaacct gcagctggat
121 cattttctga tatccattat acaaaaaaga gcgtatttat gtgcatgtat aattgcacag
181 tatgcaacca aaaatataca tatatgtttt acatgtatgt gtttctacat gccgtttgac
241 atgggagatg agggatgtta tatatagttc tgttattttc taatatgtat gtgtgttaga
301 gtgtctgtgt taatatactt tttaatgcat gctctacata atatacagta gtaataacaa
361 agagaatacg tatggaatgc gtatctctct atctatatat atatatgtat atatgctatg
```

```
 421 tgtatatcaa cctcgcatat tttctccctg ttgaccacgg ctcccacaac gtgtcgcgat
 481 ggatgacttg gcttcctatt tcgttgaaga acgcagcaaa gtgcgataag tggtatcaat
 541 tgcagaatca tttcattgat ctttgaacgC AAAcggcgca tgggagaagc tctctcgagc
 601 catcccgtg catgccacat ttctcagtgt cgaatataaa aacaaaacgc acacctattt
 661 tttgtgttgt aaaacgcacg cacaaaatcc cgccctctct tcttctcgtg tggtgcatat
721 atgtgtgtga gagtgcacac atatacgata t_tttcaactg tttctactca cacaa_tggtg
 781 tgtgacacgc atatacgtgt gtgtagagag atatggaaga gagaggaagg ggcatatata
 841 tatatacaca caaaatatat gtgtggattt gtgtgttgag cacatataag gaaaaaggtt
 901 gtgtgtatat acagagagtc tgtggcggtt gggacatgtg tataaatata tatgtatatg
 961 tgtgtgttcc gctgtggaga ttttatatct tacggagagt gttcatatat atgtttgtag
1021 gcatgtattt tggcgccccg tatagagatt aaaaaagaag agaaaaagta tgcaaaagag
1081 gcggcggata ggtgtgtatg tgtgtatcac agcaagcaac tatattttgc tgcttgtgag
1141 tatatgcata tatgtacatt atgtgcttgt gcttctttcg tgtacgcttc actttttat
1201 attgcatttt tcagacctga gtgtggcagg accacccgct aaacttaagc atattactca
 1261 gcggaggaaa agaaaacaac cg
```

***T. b. brucei* STIB215, AF306771**

```
   1 tggtgcaata caggtgatcg gaccgtcgct cgtctcgggc gaccgaaagt tcaccgatat
  61 tgcttcaata gaggaagcaa aagtcgtaac aaggtagctg taggtgaacc tgcagctgga
 121 tcattttctg atatccatta tacaaaaaag agcatattta tgtgcatgta taattgcaca
 181 gtatgcaacc aaaaatatac atatatgttt tacatgtatg tgtttctata tgccgtttga
 241 catgggagat gagggatgtt atatatagtt ctgttatttt ctaatatgta tgtgtgttag
 301 agtgtctgtg ttaatatact ttttaatgca tgctctacat aatatacagt agtaataaca
 361 aagagaatac gtatgtaatg cgtatctctc tatatatata tatatgtata tatgctatgt
 421 gtatatcaac ctccgcatat tttctccctg ttgaccacgg ctcccacaac gtgtcgcgat
 481 ggatgacttg gcttcctatt tcgttgaaga acgcagcaaa gtgcgataag tggtatcaat
```

```
 541 tgcagaatca tttcattgat ctttgaacgC AAAcggcgca tgggagaagc tctctcgagc
 601 catcccgtg catgccacat ttctcagtgt cgaatataaa aacaaaacac acacctattt
 661 tttgtgttgt tcaacgcacg cacaaaatcc cgccacctct tctcctcgtg tggtgcatat
 721 tcatgtttgt gagtgtgcac atatacgata tcattcaact gtttctactc gcacaa_tggt
 781 gtatgtcacg catatacgtg tgtgtagtga gtgatatgga agagaaatgg gaaaggcata
 841 tatatatgta tatacataat atatatgtgt gtggatttgt gtgttgagca cataggaaaa
 901 aggtgtgtgt atatacagag agtctgtggc ggttgggaca tgtgtataaa tatatatgta
 961 tatgtgtgtg ttcccctgtg gagattttat atcttacgga gagtgttcat atatatgttt
1021 gtacgcatgt attttggcgc cccgtataga gattaaaaaa gaagagaaaa agtatgcaaa
1081 agaggcgacg gatagtgtgt atgtgtgtat cacaggaaac aactatattt tgctgcttgt
1141 gagtatactg cactatatgt acattatgtg cttgtgcttc tttcagtgta cggcttcact
1201 tttttatatt gcatttttca gacctgagtg tggcaggacc acccgctaaa cttaagcata
1261 ttactcagcg gaggaaaaga aaacaaccg
```

***T. b. brucei* B8/18, AF306772**

```
   1 tggtgcaata caggtgatcg gaccgtcgct cgtctcgggc gaccgaaagt tcaccgatat
  61 tgcttcaata gaggaagcaa aagtcgtaac aaggtagctg taggtgaacc tgcagctgga
 121 tcattttctg atatccatta tacaaaaaag agcatattta tgtgcatgta taaattgcac
 181 agtatgcaac caaaaatata catatatgtt ttacatgtat gtgtttctat atgccgtttg
 241 acatgggaga tgagggatgc tatacatagt tctgttattt tctatcatgt atgtgtgtta
 301 gagtgtctgt gttaatatac tttttaatgc atgctctaca taatatacag tagtaataac
 361 acagagaata cgtatggaat gcgtatctct ctatatatat atgtgtatat atgatatgtg
 421 tatatcaacc tcgcatattt tctccctgtt gaccacggct cccacaacgt gtcgcgatgg
 481 atgacttggc ttcctatttc gttgaagaac gcagcaaagt gcgataagtg gtatcaattg
 541 cagaatcatt tcattgatct ttgaacgCAA Acggcgcatg ggagaagctc tctcgagcca
 601 tcccgtgca tgccacattt ctcagtgtcg aatataaaaa caaaacgcac acctattttg
```

```
 661 tgttgttcaa cgcacgcaaa aaatcccgcc acctcttctc ctcgtgtggt gcatattcat
 721 gtttgtgagt gtgcacatat acgatatctt tcaactgttt ctactcgcac aa_tggtgtat
 781 gtcacgcata tacgtgtgtg tagtgagtga tatggaagag aaatgggaaa ggcatatgta
 841 tatatgtata tacataatat atatgtgtgt ggatttgtgt gttgagcaca tataaggaaa
 901 aaggttgtgt gtatacacag agagtctgtg gcggttggca catgtgtata aatatatatg
 961 tatatgtgtg tgttccgctg tggagatttt atatcttacg gagacgtgtt catatatata
1021 tgtttgtacg catgtatttt ggcgccccgt atagagatta aaaaagaaga gaaaaagtat
1081 gcaaaacagg cggcggatag tgtgtatgtg tgtattcaca gcaagcaact atattttgct
1141 gcttgtgagt atatgcatat atgtacatta tgtgcttgtg cttctttcgt gtactgcttc
1201 actttttat attgtatttt tcagacctga gtgtggcagg accacccgct aaacttaagc
1261 atattactca gcggaggaaa agaaaacaac cg
```

***T. b. brucei* KP2, Clone 7, AF306773**

```
   1 tggtgcaata caggtgatcg gaccgtcgct cgtctcgggc gaccgaaagt tcaccgatat
  61 tgcttcaata gaggaagcaa aagtcgtaac aaggtagctg taggtgaacc tgcagctgga
 121 tcattttctg atatccatta tacaaaaaag agcatattta tgtgcatgta taaattgcac
 181 agtatgcaac caaaaatata catatatgtt ttacatgtat gtgtttctat atgccgtttg
 241 acatgggaga tgagggatgc tatacatagt tctgttattt tctatcatgt atgtgtgtta
 301 gagtgtctgt gttaatatac tttttaatgc atgctctaca taatatacag tagtaataac
 361 acagagaata cgtatggaat gcgtatctct ctatatatat atgtgtatat atgatatgtg
 421 tatatcaacc tcgcatattt tctccctgtt gaccacggct cccacaacgt gtcgcgatgg
 481 atgacttggc ttcctatttc gttgaagaac gcagcaaagt gcgataagtg gtatcaattg
 541 cagaatcatt tcattgatct ttgaacgCAA Acggcgcatg ggagaagctc tctcgagcca
 601 tcccgtgca tgccacattt ctcagtgtcg aatataaaaa caaaacgcac atctattttg
 661 tgttgttcaa cgcacgcaca aaatcccgcc acctcttctc ctcgtgtggt gcatattcat
 721 gtttgtgagt gtgcacatat acgatatctt tcaactcttt ctcctcgcac aa_ttgtgtat
```

```
 781 gtcacgcata tacgtgtgtg tagtgagtga tatggaagag aaatgggaaa ggcatatata
 841 tatatgtata tacataatat atatgtgtgt ggattttgtg ttgttgagca catataagaa
 901 aaaggttgtg tgtatataca gagagtctgt agcggttggg acatgtgtat aaatatatat
 961 gtatatgtgt gtgttccgct gtggagattt tatatcttac ggagagtgtt catatatata
1021 tgtttgtacg catgtatttt ggcgccccgt atagagatta aaaaagaaaa aaaaaagtat
1081 gcaaaagagg cggcggatag tgtgtatgtg tgtattcaca gcaagcaact atattttgct
1141 gcttgtgagt atatgcatat atgtacatta tgtgcttgtg cttctttcgt gtacgcttca
1201 ctttttata ttgtattttt cagacctgag tgtggcagga ccacccgcta aacttaagca
1261 tattactcag cgaaggaaaa gaaaagttcc g
```

***T. b. gambiense* Dal972 (Group I), AF306774**

```
   1 tggtgcaata caggtgatcg gaccgtcgct cgtctcgggc gaccgaaagt tcaccgatat
  61 tgcttcaata gaggaagcaa aagtcgtaac aaggtagctg taggtgaacc tgcagctgga
 121 tcattttctg atatccatta tacaaaaaag agcatattta tgtgcatgta taaattgcac
 181 agtatgcaac caaaaatata catatatgtt ttacatgtat gtgtttctat atgccgtttg
 241 acatgggaga tgagggatgc tatacatagt tctgttattt tctatcatgt atgtgtgtta
 301 gagtgtctgt gttaatatac tttttaatgc atgctctaca taatatacag tagtaataac
 361 acagagaata cgtatggaat gcgtatctct ctatatatat ttatgtatat atgctatgtg
 421 tatatcaacc tcgcatattt tctccctgtt gaccacggct cccacaacgt gtcgcgatgg
 481 atgacttggc ttcctatttc gttgaagaac gcagcaaagt gcgataagtg gtatcaattg
 541 cagaatcatt tcattgCCCA atctttgaac gcaaacggcg catgggagaa gctctctcga
 601 gccatcccg tgcatgccac atttctcagt gtcgaatata aaaacaaaac acacacctat
 661 tttgtgttgt tcaacgcacg caaaaaatcc cgccacctct ttccttggg ggggcatat
 721 tcatgtttga gtgtgcacaa acacgatatc tttcaactct ttatactcgc acaatggtgt
 781 atgtcacgca tatacgtgtg tgtagtgagt gatatggaag agaaatggga gaggcatatg
 841 tatatatgta tatacataat atatatgtgt gtggatttgt gtgttgagca catataagga
```

```
 901 aaaaggttgt gtgtatatac agagagtctg tggcggttgg gacatgtgta taaatatata
 961 tgtatatgtg tgtgttccgc tgtggagatt ttatatctta cggagagtgt tcatagatat
1021 atgtttgaac gcatgtattt tggcgccccg tatagagatt aaaaaagaag agaaaaagta
1081 tgcaaaagag gcggcggata gtgtgtatgt gtgtattcac agcaagcaac tatattttgc
1141 tgcttgtgag tatatgcata tatgtacatt atgtgcttgt gcttctttcg tgtacgcttc
1201 actttttat attgtatttt tcagacctga gtgtggcagg accacccgct aaacttaagc
1261 atattactca gcggaggaaa agaaaacaac cg
```

***T. b. gambiense* Suzena (Group I), AF306775**

```
   1 tggtgcaata caggtgatcg gaccgtcgct cgtctcgggc gaccgaaagt tcaccgatat
  61 tgcttcaata gaggaagcaa aagtcgtaac aaggtagctg taggtgaacc tgcagctgga
 121 tcattttctg atatccatta tacaaaaaag agcatattta tgtgcatgta taaattgcac
 181 agtatgcaac caaaaatata catatatgtt ttacatgtat gtgtttctat atgccgtttg
 241 acatgggaga tgagggatgc tatacatagt tctgttattt tctatcatgt atgtgtgtta
 301 gagtgtctgt gttaatatac tttttaatgc atgctctaca taatatacag tagtaataac
 361 acagagaata cgtatggaat gcgtatctct ctatatatat ttatgtatat atgctatgtg
 421 tatatcaacc tcgcatattt tctccctgtt gaccacggct cccacaacgt gtcgcgatgg
 481 atgacttggc ttcctatttc gttgaagaac gcagcaaagt gcgataagtg gtatcaattg
 541 cagaatcatt tcattgCCCA atctttgaac gcaaacggcg catgggagaa gctctctcga
 601 gccatcccg tgcatgccac atttctcagt gtcgaatata aaaacaaaac acacacctat
 661 tttgtgttgt tcaacgcacg caaaaaatcc cgccacctct tctcctcgtg tgggcatat
721 tcatgtttga gtgtgcacaa acacgatatc tttgcaactc tttctactcg
caca  atg gta
 781 tgtcacgcat atacgtgtgt gtagtgagtg atatggaaga aatatgggat aaggcataag
 841 tatatatgta taaacatgat atatatgtgt gtggatctgt gtgttgagca catataagga
 901 aaaagggtgt gtgtatatac agagagtctg tggcggttgg gacatgtgta taaatatata
```

```
 961 tgtatatgtg tgtgttccgc tgtggagatt ttatatctta cggagagtgt tcatatatat
1021 atgtttgtac gcatgtattt tggcgccccg tatagagatt aaaaaagaag agaaaaagta
1081 tgcaaaagag gcggcggata gtgtgtatgt gtgtattcac aggcaagcaa ctatattttg
1141 ctgcttgtga gtatatgcat atatgtacat tatgtgcttg tgcttctttc agtgtactgc
1201 ttcacttttt tatattgtat ttttcagacc tgagtgtggc aggaccaccc gctaaactta
1261 agcatattac tcagcggagg aaaagaaaac aaccg
```

***T. b. gambiense* NW2 (Group I), AF306776**

```
   1 tggtgcatac aggtgatcgg accgtcgctc gtctcgggcg accgaaagtt caccgatatt
  61 gcttcaatag aggaagcaaa agtcgtaaca aggtagctgt aggtgaacct gcagctggat
 121 cattttctga tatccattat acaaaaaaga gcatatttat gtgcatgtat aaattgcaca
 181 gtatgcaacc aaaaatatac atatatgttt tacatgtatg tgtttctata tgccgtttga
 241 catgggagat gagggatgct atacatagtt ctgttatttt ctatcatgta tgtgtgttag
 301 agtgtctgtg ttaatatact ttttaatgca tgctctacat aatatacagt agtaataaca
 361 cagagaatac gtatggaatg cgtatctctc tatatatatt tatgtatata tgatatgtgt
 421 atatcaacct cgcatatttt ctccctgttg accacggctc ccacaacgtg tcgcgatgga
 481 tgacttggct tcctatttcg ttgaagaacg cagcaaagtg cgataagtgg tatcaattgc
 541 agaatcattt cattgCCCAa tctttgaacg caaacggcgc atgggagaag ctctctcgag
 601 ccatcccgt gcatgccaca tttctcagtg tcgaatataa aaacaaaaca cacacctatt
 661 ttgtgttgtt caacgcacgc aaaaaatccc gccacctctt cttctcgtgt ggggcatatt
 721 catgtttgtg agtgtgcaca tatacgatat ctttcagctc tttctactcg cacaa tggtg
 781 tatgtcacgc atatacgtgt gtgtagtgag tgatatgcaa cagaaatggg aaaggcatat
 841 gtatatatgt atatacataa tatatatgtg tgtggatttg tgtgttgagc acatataagg
 901 aaaaaggttg tgtgtatata cagagagtct gtggcggttg ggacatgtgt ataaatatat
 961 atgtatatgt gtgtgttccg ctgtggagat tttatatctt acggagagtg ttcatatata
1021 tatgtttgta cgcatgtatt ttggcgcccc gtatagagat taaaaaagaa gagaaaaagt
```

```
1081 atgcaaaaga ggcggcggat agtgtgtatg tgtgtattca cagcaagcaa ctatattttg
1141 ctgcttgtga gtatatgcat atatgtacat tatgtgcttg tgcttctttc gtgtacgctt
1201 cacttttta tattgtattt ttcagacctg agtgtggcag gaccacccgc taaacttaag
1261 catattactc agcggaggaa aagaaaacaa ccg
```

***T. b. gambiense* TH2 (Grp II), AF306777**

```
   1 tggtgcaata caggtgatcg gaccgtcgct cgtctcgggc gaccgaaagt tcaccgatat
  61 tgcttcaata gaggaagcaa aagtcgtaac aaggtagctg taggtgaacc tgcagctgga
 121 tcattttctg atatccatta tacaaaaaag agcatattta tgtgcatgta taaattgcac
 181 agtatgcaac caaaaatata catatatgtt ttacatgtat gtgtttctat atgccgtttg
 241 acatgggaga tgagggatgc tatacatagt tctgttattt tctatcatgt atgtgtgtta
 301 gagtgtctgt gttaatatac tttttaatgc atgctctaca taatatacag tagtaataac
 361 acagagaata cgtatggaat gcgtatctct ctatatatat ttatgtatat atgatatgtg
 421 tatatcaacc tcgcatattt tctccctgtt gaccacggct cccacaacgt gtcgcgatgg
 481 atgacttggc ttcctatttc gttgaagaac gcagcaaagt gcgataagtg gtatcaattg
 541 cagaatcatt tcattgCCCA atctttgaac gcaaacggcg catgggagaa gctctctcga
 601 gccatcccg tgcatgccac atttctcagt gtcgaatata aaaacaaaac gcacacctat
 661 tttgtgttgt tcaacgcacg caaaaaatcc cgccacctct tctcctcgtg tgggcatat
 721 tcatgtttgt gagtgtgcac atatacgata tctttcatct ctttcttctc gcacatattg
 781 tgtatgtcac gcatatacgt gtgtgtagtg agtgatatga aagagaaatg ggaaaggcat
 841 atgtatatat gtatacacat aatatatatg tgtgtggatt tgtgtgttga gcacatataa
 901 ggaaaaaggt tgtgtgtata cacagagagt ctgtggcggt tgggacatgt gtataaatat
 961 atatgtatat gtgtgtgttc cgctgtggag atttatattc ttacggagag tgttcatata
1021 tatatgtttg aacgcatgta ttttggcgcc ccgtatagag attaaaaaag aagaggaaaa
1081 gtatgcaaaa gaggcggcgg atagtgtgta tgtgtgtatt cacagcaagc aactatattt
1141 tgctgcttgt gagtatatgc atatatgtac attatgtgct tgtgcttctt tcgtgtacgc
```

```
1201 ttcacttttt tatattgtat ttttcagacc tgagtgtggc aggaccaccc gctaaactta

1261 agcatattac tcagcggagg aaaagaaaac aacctg
```

Appendix 14. LAMP primer sets generated by Primer Explorer V4 for the *TgsGP* gene.

Target DNA		AACATGTCGG	CAGCGGGACC	GGATTGGAGA	AACCAGTTCG	TCAGCAGCAA	AGGTGTTAAG	CAGGAATGGG
(Complement)		ttgtacagcc	gtcgccctgg	cctaacctct	ttggtcaagc	agtcgtcgtt	tccacaattc	Gtccttaccc
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	251	261	271	281	291	301	311
[22]	-1.12							
[59]	-2.32							
[11]	-1.67	[11]	CGGGACC	GGATTGGAGA	A GTTCG	TCAGCAGCAA	AGGT	
[18]	-2.12			[18]	CG	TCAGCAGCAA	AGGTGT	G
[28]	-1.52							
Target DNA		AGCCAACAGC	GAAGAACAAA	GCCGTAGCGG	AGGCATGGGC	GCGTACGTAC	GCGGGATGGA	TCAACACAGC
(Complement)		tcggttgtcg	cttcttgttt	cggcatcgcc	tccgtacccg	cgcatgcatg	cgccctacct	Agttgtgtcg
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	321	331	341	351	361	371	381
[22]	-1.12						[22]	CAGC
[59]	-2.32							
[11]	-1.67	tcg	cttcttgttt	cggcatcgc		AC	GCGGGATGGA	TCAACACAG
[18]	-2.12	AGCCAACAGC	GAAGAACA			gcatgcatg	cgccctacct	ag GC
[28]	-1.52							
Target DNA		ACTCGTCCTT	TATGCAGGCG	GCAGCGACGA	CAGGAAACGA	GCGATCAGCA	AATTTGACAG	CATGGGAGAT
(Complement)		tgagcaggaa	atacgtccgc	cgtcgctgct	gtcctttgct	cgctagtcgt	ttaaactgtc	Gtaccctcta
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	391	401	411	421	431	441	451

[22]	-1.12	ACTCGTCCTT	TATGCA	CAGCGACGA	CAGGAAACG			
[59]	-2.32							
[11]	-1.67				cctttgct	cgctagtcgt		Ctcta
[18]	-2.12	ACTCGTCCTT	TATGCAGGCG				aactgtc	Gtaccctcta
[28]	-1.52		[28]	A	CAGGAAACGA	GCGATCAG	CAG	CATGGGAGAT
Target DNA		GCAACTCGCA	AGCTAGCACA	GCGGAAGCTG	GAAGCCATTT	TGGCAAAAGT	GCAGCCGCTG	AGAAGCAAGC
(Complement)		cgttgagcgt	tcgatcgtgt	cgccttcgac	cttcggtaaa	accgttttca	cgtcggcgac	tcttcgttcg
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	461	471	481	491	501	511	521
[22]	-1.12	gcgt	tcgatcgtgt	cgcctt			CAGCCGCTG	AGAAGCAAGC
[59]	-2.32							
[11]	-1.67	cgttgagcgt	tcga	[11]				
[18]	-2.12	cg		ccttcgac	cttcggtaaa	ac	[18]	
[28]	-1.52	GCAACTC			taaa	accgttttca	cgtcggcgTG	AGAAGCAAGC
Target DNA		TCAGTGCACT	TAAAGCGGTA	GTAGAAGCGG	GAACGGGCAA	GGCTGTCACG	GACTTGCTCA	AGGCGGCGCT
(Complement)		agtcacgtga	atttcgccat	catcttcgcc	cttgcccgtt	ccgacagtgc	ctgaacgagt	tccgccgcga
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	531	541	551	561	571	581	591
[22]	-1.12	TC				acagtgc	ctgaacgagt	tc a

[59]	-2.32							
[11]	-1.67							
[18]	-2.12							
[28]	-1.52	TCAGTGCACT				cagtgc	ctgaacgagt	tcc a
Target DNA		CTACGGCGGT	ATCGACGGCG	GCAGTGACTT	CGAGGACGCG	ACAAAGGACA	AGGACGGCGA	ACGTGTGCGA
(Complement)		gatgccgcca	tagctgccgc	cgtcactgaa	gctcctgcgc	tgtttcctgt	tcctgccgct	tgcacacgct
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	601	611	621	631	641	651	661
[22]	-1.12	gatgccgcca	tagctgc	[22]				
[59]	-2.32							
[11]	-1.67							
[18]	-2.12							
[28]	-1.52	gatgccgcca	tagctgc	[28]				
Target DNA		ACGTGTGCGA	GGCATCTGCA	AGGCCGCCGG	AAAAGTGAAG	GGTAACCAGA	CACTGGCAGA	TGTGCTGCTT
(Complement)		tgcacacgct	ccgtagacgt	tccggcggcc	ttttcacttc	ccattggtct	gtgaccgtct	acacgacgaa
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	661	671	681	691	701	711	721
[22]	-1.12							

[59]	-2.32							
[11]	-1.67							
[18]	-2.12							
[28]	-1.52							
Target DNA		TGCGTATGCG	ATACCGCAGT	AAGCTACGGC	GACGACGGCA	ACAAAAAAAT	TTGTGCCAAA	CTGAGCGGTA
(Complement)		acgcatacgc	tatggcgtca	ttcgatgccg	ctgctgccgt	tgtttttta	aacacggttt	gactcgccat
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	731	741	751	761	771	781	791
[22]	-1.12							
[59]	-2.32							
[11]	-1.67							
[18]	-2.12							
[28]	-1.52							
Target DNA		AGGCCGGAGC	GAAGCAGTGG	GACCTTAGCG	ACAGGGGCGA	TGTCGCTGCT	GTGTTCGGAG	AGCTCAGACA
(Complement)		tccggcctcg	cttcgtcacc	ctggaatcgc	tgtccccgct	acagcgacga	cacaagcctc	tcgagtctgt
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	801	811	821	831	841	851	861
[22]	-1.12							

[59]	-2.32	[59]	GAAGCAGTGG	GACCTTAGC	CGA	TGTCGCTGCT	GTGTTC	
[11]	-1.67							
[18]	-2.12							
[28]	-1.52							
Target DNA		GGGCTGTAAT	AGCAAGCAAG	AGCACAAAAC	CACAGCAGGC	GGGATCAGGG	CGGCCCTGGC	GGCGATAAGG
(Complement)		cccgacatta	tcgttcgttc	tcgtgttttg	gtgtcgtccg	ccctagtccc	gccgggaccg	ccgctattcc
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	871	881	891	901	911	921	931
[22]	-1.12							
[59]	-2.32			ttttg	gtgtcgtccg	ccctag		
[11]	-1.67							
[18]	-2.12							
[28]	-1.52							
Target DNA		AGCAAATTCC	AAATTGACGG	GGACAACGGC	TATCTAGGAA	GGTACGACAC	CGACGGCAAC	TGCACAGGAA
(Complement)		tcgtttaagg	tttaactgcc	cctgttgccg	atagatcctt	ccatgctgtg	gctgccgttg	acgtgtcctt
CONSENSUS(*)		**********	**********	**********	**********	**********	**********	**********
Primer	dG(dimer)	941	951	961	971	981	991	1001
[22]	-1.12							

[59]	-2.32	TCC	AAATTGACGG	GGACAACGG			gttg	acgtgtcctt
[11]	-1.67							
[18]	-2.12							
[28]	-1.52							
Target DNA		CGGCGCCAGG	CGGTGTCTGC	GTTAAATATG	CCGGCTACGG			
(Complement)		gccgcggtcc	gccacagacg	caatttatac	ggccgatgcc			
CONSENSUS(*)		**********	**********	**********	**********			
Primer	dG(dimer)	1011	1021	1031	1041			
[22]	-1.12							
[59]	-2.32	gccg tcc	gccacagacg	caatt	[59]			
[11]	-1.67							
[18]	-2.12							
[28]	-1.52							

Appendix 15. Binding sites of the *TgsGP* nested PCR primers and the LAMP *TgsGP* primers on the *TgsGP* gene

Table 1. Key to Figure 1

Reaction	Primer	Binding site on the *TgsGP* gene
PCR	TgsGPf1	719 - 738
	TgsGPr1	1220 - 1241
	TgsGPf2	845 - 863
	TgsGPr2	1134 - 1152
LAMP	FIP-F1c	896 - 916
	FIP-F2	838 - 856
	BIP-B1c	948 - 969
	BIP-B2	997 - 1014
	LF	855 - 875
	LB	970 - 992
	F3	811 - 829
	B3	1018 -1035

Figure 1a. Binding sites of the *TgsGP* nested PCR primers on the *TgsGP* gene (Accession number AJ277951)

```
   1 atctcgacgg acttttgtaa ttttcaaaag aagcagagaa agctaactaa tacgagaaat
  61 gtggcaatta ctagcaatag cggcggcgct agcactttca tcacggccat cagacggaga
 121 ggacagtgca ggcgaaaatg gcggcacgta cgcagccctc tgtacactgc taacagaagc
```

```
 181 acttggtgaa gttgaccaag cgcagcccac taaaggatgg gaacaggcat atgcttctat
 241 tcttgaagcc aacatgtcgg cagcgggacc ggattggaga aaccagttcg tcagcagcaa
 301 aggtgttaag caggaatggg agccaacagc gaagaacaaa gccgtagcgg aggcatgggc
 361 gcgtacgtac gcgggatgga tcaacacagc actcgtcctt tatgcaggcg gcagcgacga
 421 caggaaacga gcgatcagca aatttgacag catgggagat gcaactcgca agctagcaca
 481 gcggaagctg gaagccattt tggcaaaagt gcagccgctg agaagcaagc tcagtgcact
 541 taaagcggta gtagaagcgg gaacgggcaa ggctgtcacg gacttgctca aggcggcgct
 601 ctacggcggt atcgacggcg gcagtgactt cgaggacgcg acaaaggaca aggacggcga
 661 acgtgtgcga ggcatctgca aggccgccgg aaaagtgaag ggtaaccaga cactggcaga
 721 tgtgctgctt tgcgtatgcg ataccgcagt aagctacggc gacgacggca acaaaaaaat
 781 ttgtgccaaa ctgagcggta aggccggagc gaagcagtgg gaccttagcg acaggggcga
 841 tgtcgctgct gtgttcggag agctcagaca gggctgtaat agcaagcaag agcacaaaac
 901 cacagcaggc gggatcaggg cggccctggc ggcgataagg agcaaattcc aaattgacgg
 961 ggacaacggc tatctaggaa ggtacgacac cgacggcaac tgcacaggaa cggcgccagg
1021 cggtgtctgc gttaaatatg ccggctacgg caccaacact gggaacggtt ggcacgatat
1081 tcaatgggtc aggcatgcaa cggccgcagc tgcagcaatt gaggcaggag cccgagcggc
1141 aagcacgatg gcagcgctgg agcccctgct agaagcagcg gcggttgaag cttgggaagt
1201 ggcaaacacc acagcaagca attcttatgt gatgaaggcc cctcttttgc ttgcattttt
1261 gctttttaa aattttcccc tatttaaaga actttgctat ttgatatatt ttaacac
```

Figure 1b. Binding sites of the LAMP *TgsGP* primers on the *TgsGP* gene (Accession number AJ277951)

```
   1 atctcgacgg acttttgtaa ttttcaaaag aagcagagaa agctaactaa tacgagaaat
  61 gtggcaatta ctagcaatag cggcggcgct agcactttca tcacggccat cagacggaga
 121 ggacagtgca ggcgaaaatg gcggcacgta cgcagccctc tgtacactgc taacagaagc
```

```
 181 acttggtgaa gttgaccaag cgcagcccac taaaggatgg gaacaggcat atgcttctat
 241 tcttgaagcc aacatgtcgg cagcgggacc ggattggaga aaccagttcg tcagcagcaa
 301 aggtgttaag caggaatggg agccaacagc gaagaacaaa gccgtagcgg aggcatgggc
 361 gcgtacgtac gcgggatgga tcaacacagc actcgtcctt tatgcaggcg gcagcgacga
 421 caggaaacga gcgatcagca aatttgacag catgggagat gcaactcgca agctagcaca
 481 gcggaagctg gaagccattt tggcaaaagt gcagccgctg agaagcaagc tcagtgcact
 541 taaagcggta gtagaagcgg gaacgggcaa ggctgtcacg gacttgctca aggcggcgct
 601 ctacggcggt atcgacggcg gcagtgactt cgaggacgcg acaaaggaca aggacggcga
 661 acgtgtgcga ggcatctgca aggccgccgg aaaagtgaag ggtaaccaga cactggcaga
 721 tgtgctgctt tgcgtatgcg ataccgcagt aagctacggc gacgacggca acaaaaaaat
 781 ttgtgccaaa ctgagcggta aggccggagc gaagcagtgg gaccttagcg acagggcga
 841 tgtcgctgct gtgttcggag agctcagaca gggctgtaat agcaagcaag agcacaaaac
 901 cacagcaggc gggatcaggg cggccctggc ggcgataagg agcaaattcc aaattgacgg
 961 ggacaacggc tatctaggaa ggtacgacac cgacggcaac tgcacaggaa cggcgccagg
1021 cggtgtctgc gttaaatatg ccggctacgg caccaacact gggaacggtt ggcacgatat
1081 tcaatgggtc aggcatgcaa cggccgcagc tgcagcaatt gaggcaggag cccgagcggc
1141 aagcacgatg gcagcgctgg agcccctgct agaagcagcg gcggttgaag cttgggaagt
1201 ggcaaacacc acagcaagca attcttatgt gatgaaggcc cctcttttgc ttgcattttt
1261 gctttttaa aattttcccc tatttaaaga actttgctat ttgatatatt ttaacac
```

Appendix 16. Results from the *TgsGP* LAMP assay applied to the total sample set

Table 1. *TgsGP* LAMP in the real time turbidimeter, for all 86 DNA samples

Sample	[DNA] (ng μl^{-1})	TBR PCR	*TgsGP* PCR	LAMP *PfrA* (Tt)	LAMP *TgsGP* (Tt)
1. Tira 24	6.48	+	-	00:37:24	00:00:00
2. Sikuda 28	47.25	+	-	00:37:36	00:00:00
3. UGC	0.01	+	-	00:35:12	00:00:00
4. UGI	1.01	+	-	00:36:36	00:00:00
5. Dal069 IM52	73.49	+	+	00:36:48	00:37:30
6. Papol 33	21.96	+	-	00:35:54	00:00:00
7. Bumanda 146	60.14	+	-	00:36:00	00:00:00
8. Papol 264	69.57	+	-	00:36:24	00:00:00
9. UGA 88	22.53	+	-	00:45:36	00:00:00
10. E. Oketch	15.21	+	-	00:50:06	00:00:00
11. Papol 285	12.12	+	-	00:48:18	00:00:00
12. Biyamina	26.87	+	-	00:47:54	01:05:36
13. Mela 3	24.09	+	-	00:48:54	00:00:00
14. Papol 103	5.77	+	-	00:51:00	00:00:00
15. Mawero 66	18.37	+	-	00:49:18	00:00:00
16. Mela 71 IM87	23.3	+	-	00:48:06	00:00:00

17. TH149	20.31	+	+	00:42:42	00:40:00
18. Bida 3 Clone A	53.14	-	+	00:46:12	00:50:54
19. TSW83	27.45	+	-	00:40:06	00:00:00
20. Katerema 311	7.39	+	+	00:46:00	00:00:00
21. Musikia Clone A	5.72	-	+	00:52:24	00:00:00
22. Suzena	8.13	+	+	00:52:48	00:00:00
23. Tsuaa	14.77	+	+	00:49:00	00:54:54
24. Mawero 32	4.62	+	-	00:43:36	00:00:00
25. Papol 42	1.7	+	-	00:34:36	00:00:00
26. UGK	9.57	+	-	00:33:12	00:00:00
27. Papol 60	0.32	+	-	00:31:54	00:00:00
28. UR4	49.11	+	-	00:32:36	00:00:00
29. F97	2.75	+	-	00:32:12	00:00:00
30. Mawero 80	5.49	+	-	00:33:24	00:00:00
31. Papol 278	7.64	+	-	00:42:00	00:00:00
32. URI	18.15	+	-	00:40:54	00:00:00
33. UGH	1.87	+	+	00:29:18	00:00:00
34. Mela Pig	7.19	+	-	00:30:36	00:00:00
35. Tira 22	4.44	+	-	00:29:00	00:00:00
36. Mela 66	6.01	+	-	00:29:24	00:00:00
37. Uganda B	8.66	+	-	00:28:00	00:00:00

38. Mela 24	4.34	+	-	00:28:42	00:00:00
39. Iyolowa 125	13.15	+	-	00:28:54	00:00:00
40. Tira 168	4.98	+	-	00:27:42	00:00:00
41. Papol 12	1.45	+	-	00:31:12	00:00:00
42. Mela 27	5	+	-	00:31:30	00:00:00
43. Poyem 11	10.6	+	-	00:31:36	00:00:00
44. Tira 4	5.13	+	-	00:31:06	00:00:00
45. Mela pig 2	17.89	+	-	00:30:18	00:00:00
46. UGE	6.05	+	-	00:28:48	00:00:00
47. Katerema 72	1.92	+	-	00:31:54	00:00:00
48. Mawero 42	7.92	+	-	00:30:36	00:00:00
49. Tira 34	1.71	+	-	00:38:18	01:05:24
50. H.Taka	20.26	+	-	00:29:42	00:00:00
51. Uganda A	9.04	+	-	00:30:30	00:00:00
52. A Kiburige	26.28	+	-	00:31:54	00:00:00
53. Uganda E	6.04	+	-	00:30:42	00:00:00
54. Katerema 116	14.12	+	-	00:30:24	00:00:00
55. Tira 43	5.81	+	-	00:30:42	00:00:00
56. Tira 92	13.14	+	-	00:00:00	00:00:00
57. Tira 75	4.93	+	-	00:36:30	00:00:00
58. Musikia Clone B	9.11	+	+	00:30:00	00:58:24

59. Iyolowa 116	3.94	+	-	00:25:48	00:00:00
60. Iyolowa 153	5.36	+	-	00:27:48	00:00:00
61. TH 112 IM56	21.32	+	-	00:29:00	00:00:00
62. Muraz 15 IM47	24.87	+	-	00:00:00	00:00:00
63. Mela 12	6.78	+	-	00:35:48	00:00:00
64. Mawero 31	24.32	+	-	00:33:54	00:00:00
65. F73	7.7	+	-	00:49:42	00:00:00
66. Tira 27	30.69	+	-	00:53:54	00:00:00
67. Uganda L	3.19	+	-	00:46:54	00:00:00
68. F48	8.76	+	-	00:47:12	00:00:00
69. Tira 68	11.96	+	-	00:47:30	00:00:00
70. Tira 30	1.52	+	-	00:48:00	00:00:00
71. Tira 29	1.55	-	-	00:00:00	00:00:00
72. Papol 371	144.78	+	-	00:00:00	00:00:00
73. Mawero 85	6.83	+	-	00:49:00	00:00:00
74. Iyolowa 147	1.33	+	-	00:45:54	00:00:00
75. Muraz 3 IM46	29.59	+	-	00:46:00	00:00:00
76. Katerema 41	5.51	+	-	00:47:24	00:00:00
77. UGG 88	24.29	+	-	00:47:54	00:00:00
78. Mela 2	3.73	+	-	00:49:06	01:27:36
79. Uganda M	2.17	+	-	00:52:06	00:00:00

80. Rose Akinare	2.39	+	-	00:48:18	00:00:00
81. Uganda B	0.79	+	-	00:44:54	00:00:00
82. Papol 144	2.88	+	-	00:46:12	00:00:00
83. Uganda C	9.76	+	-	00:47:00	00:00:00
84. Sikuda 42.90	8.99	+	-	00:44:42	00:00:00
85. Sikuda 4	6.3	-	-	00:00:00	00:00:00
86. D. Obwang	8.2	+	-	00:41:12	01:24:18

Table 2. *TgsGP* LAMP, in triplicate for all 86 Trypanosome DNA samples, assessed by turbidity and gel electrophoresis

	Run 1		Run 2		Run 3	
Sample	**Turbidity**	**Gel**	**Turbidity**	**Gel**	**Turbidity**	**Gel**
1. Tira 24	-	-	-	-	-	-
2. Sikuda 28	-	-	-	-	-	-
3. UGC	-	-	-	-	-	-
4. UGI	-	-	-	-	-	-
5. Dal069 IM52	+	+	+	+	+	+
6. Papol 33	-	-	-	-	-	-
7. Bumanda 146	-	-	-	-	-	-
8. Papol 264	-	-	-	-	-	-
9. UGA 88	-	-	-	-	-	-
10. E. Oketch	-	-	-	-	-	-

11. Papol 285	-	-	-	-	-	-
12. Biyamina	-	-	-	-	-	-
13. Mela 3	-	-	-	-	-	-
14. Papol 103	-	-	-	-	-	-
15. Mawero 66	-	-	-	-	-	-
16. Mela 71 IM87	-	-	-	-	-	-
17. TH149	+	+	-	-	+	+
18. Bida 3 Clone A	+	+	+	+	+	+
19. TSW83	-	-	-	-	-	-
20. Katerema 311	-	+	-	+	+	+
21. Musikia Clone A	+	+	-	+	+	+
22. Suzena	-	+	-	-	+	+
23. Tsuaa	+	+	+	+	+	+
24. Mawero 32	-	-	-	-	-	-
25. Papol 42	-	-	-	-	-	-
26. UGK	-	-	-	-	-	-
27. Papol 60	-	-	-	-	-	-
28. UR4	-	-	-	-	-	-
29. F97	-	-	-	-	-	-
30. Mawero 80	-	-	-	-	-	-

31. Papol 278	-	-	-	-	-	-
32. URI	-	-	-	-	-	-
33. UGH	-	-	-	-	-	-
34. Mela Pig	-	-	-	-	-	-
35. Tira 22	-	-	-	-	-	-
36. Mela 66	-	-	-	-	-	+
37. Uganda B	-	-	-	-	-	-
38. Mela 24	-	-	-	-	-	-
39. Iyolowa 125	-	-	-	-	-	-
40. Tira 168	-	-	-	-	-	-
41. Papol 12	-	-	-	-	-	-
42. Mela 27	-	-	-	-	-	-
43. Poyem 11	-	-	-	-	-	-
44. Tira 4	-	-	-	-	-	-
45. Mela pig 2	-	-	-	-	-	-
46. UGE	-	-	-	-	-	-
47. Katerema 72	-	-	-	-	-	-
48. Mawero 42	-	-	-	-	-	-
49. Tira 34	-	-	+	+	-	-
50. H.Taka	-	-	-	-	-	-
51. Uganda A	-	-	-	-	+	+

52. A Kiburige	-	-	-	-	-	-
53. Uganda E	-	-	-	-	+	+
54. Katerema 116	-	-	-	-	-	-
55. Tira 43	-	-	+	+	-	-
56. Tira 92	-	-	-	-	-	-
57. Tira 75	-	-	-	-	-	-
58. Musikia Clone B	-	+	+	+	+	+
59. Iyolowa 116	-	-	-	-	-	-
60. Iyolowa 153	-	-	-	-	-	-
61. TH 112 IM56	-	-	-	-	-	-
62. Muraz 15 IM47	-	-	-	-	+	+
63. Mela 12	-	-	-	-	+	+
64. Mawero 31	-	-	-	+	-	-
65. F73	-	-	-	+	-	+
66. Tira 27	-	-	-	-	-	-
67. Uganda L	-	-	-	-	-	-
68. F48	-	-	+	+	-	+
69. Tira 68	-	-	-	-	-	-
70. Tira 30	-	-	-	-	-	-
71. Tira 29	-	-	-	-	-	-

72. Papol 371	-	-	-	-	+	+
73. Mawero 85	-	-	-	-	-	-
74. Iyolowa 147	-	-	-	-	-	-
75. Muraz 3 IM46	-	-	-	-	-	-
76. Katerema 41	-	-	-	-	-	-
77. UGG 88	-	-	-	-	-	-
78. Mela 2	-	-	-	-	+	+
79. Uganda M	-	-	-	-	-	-
80. Rose Akinare	-	-	-	-	-	-
81. Uganda B	-	-	-	-	-	-
82. Papol 144	-	-	-	-	-	-
83. Uganda C	-	-	-	-	-	-
84. Sikuda 42.90	-	-	-	-	-	-
85. Sikuda 4	-	-	-	-	-	-
86. D. Obwang	-	-	-	-	-	-

Appendix 17. All PCR and LAMP assay results.

Table 1. PCR assay results for the 86 DNA samples

Sample	TBR PCR	*TgsGP* PCR	*SRA* PCR			Sample	TBR PCR	*TgsGP* PCR	*SRA* PCR		
			VSG	*SRA*	*GPI-PLC*				*VSG*	*SRA*	*GPI-PLC*
1. Tira 24	+	-	+	-	+	44. Tira 4	+	-	+	-	+
2. Sikuda 28	+	-	+	-	+	45. Mela pig 2	+	-	+	-	+
3. UGC	+	-	+	+	+	46. UGE	+	-	+	+	+
4. UGI	+	-	+	+	+	47. Katerema 72	+	-	+	+	+
5. Dal069 IM52	+	+	+	+	+	48. Mawero 42	+	-	+	+	+
6. Papol 33	+	-	+	-	+	49. Tira 34	+	-	+	-	+
7. Bumanda 146	+	-	+	-	+	50. H.Taka	+	-	+	+	+
8. Papol 264	+	-	+	-	+	51. Uganda A	+	-	+	+	+
9. UGA 88	+	-	+	+	+	52. A Kiburige	+	-	+	+	+
10. E. Oketch	+	-	+	+	+	53. Uganda E	+	-	+	+	+

11. Papol 285	+	-	+	+	+	54. Katerema 116	+	-	+	+	+
12. Biyamina	+	-	+	+	+	55. Tira 43	+	-	+	-	+
13. Mela 3	+	-	+	-	+	56. Tira 92	+	-	-	-	-
14. Papol 103	+	-	+	+	+	57. Tira 75	+	-	+	-	+
15. Mawero 66	+	-	+	+	+	58. Musikia Clone B	+	+	-	-	+
16. Mela 71 IM87	+	-	+	+	+	59. Iyolowa 116	+	-	+	-	+
17. TH149	+	+	+	-	+	60. Iyolowa 153	+	-	+	-	+
18. Bida 3 Clone A	-	+	+	-	+	61. TH 112 IM56	+	-	+	-	+
19. TSW83	+	-	+	-	+	62. Muraz 15 IM47	+	-	+	-	+
20. Katerema 311	+	+	+	-	+	63. Mela 12	+	-	+	-	+
21. Musikia Clone A	-	+	-	-	+	64. Mawero 31	+	-	+	+	+
22. Suzena	+	+	-	-	+	65. F73	+	-	+	+	+
23. Tsuaa	+	+	-	-	+	66. Tira 27	+	-	+	-	+
25. Papol 42	+	-	+	-	+	68. F48	+	-	+	+	+
26. UGK	+	-	+	+	+	69. Tira 68	+	-	+	-	+

27. Papol 60	+	-	+	-	+	70. Tira 30	+	-	+	-	+
28. UR4	+	-	+	-	+	71. Tira 29	-	-	+	-	+
29. F97	+	-	+	-	+	72. Papol 371	+	-	+	-	+
30. Mawero 80	+	-	+	-	+	73. Mawero 85	+	-	+	-	+
31. Papol 278	+	-	+	+	+	74. Iyolowa 147	+	-	+	-	+
32. URI	+	-	+	+	+	75. Muraz 3 IM46	+	-	+	-	+
33. UGH	+	+	+	+	+	76. Katerema 41	+	-	+	-	+
34. Mela Pig	+	-	+	-	+	77. UGG 88	+	-	+	+	+
35. Tira 22	+	-	+	-	+	78. Mela 2	+	-	+	+	+
36. Mela 66	+	-	+	+	+	79. Uganda M	+	-	+	+	+
37. Uganda B	+	-	+	+	+	80. Rose Akinare	+	-	+	+	+
38. Mela 24	+	-	+	-	+	81. Uganda B	+	-	+	+	+
39. Iyolowa 125	+	-	+	-	+	82. Papol 144	+	-	+	-	+
40. Tira 168	+	-	+	-	+	83. Uganda C	+	-	+	+	+
41. Papol 12	+	-	+	-	+	84. Sikuda 42.90	+	-	+	-	+

42. Mela 27	+	-	+	+	+	85. Sikuda 4	-	-	-	-	-
43. Poyem 11	+	-	+	-	+	86. D. Obwang	+	-	+	+	+

Table 2. LAMP assay results for the 86 DNA samples

Sample	LAMP RIME					LAMP *PfrA*					LAMP *TgsGP*					LAMP *SRA*1					SRALAMP_a				
	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v
1. Tira 24	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-
2. Sikuda 28	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
3. UGC	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
4. UGI	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
5. Dal069 IM52	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+
6. Papol 33	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
7. Bumanda 146	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8. Papol 264	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9. UGA 88	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
10. E. Oketch	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
11. Papol 285	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
12. Biyamina	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		

13. Mela 3	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
14. Papol 103	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
15. Mawero 66	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
16. Mela 71 IM87	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
17. TH149	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-
18. Bida 3 Clone A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-
19. TSW83	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
20. Katerema 311	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
21. Musikia Clone A	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-
22. Suzena	+	+	+			+	+	+			+	-	+			-	-	-			-	-	-		
23. Tsuaa	+	+	+			+	+	+			+	+	+			-	-	-			-	-	-		
24. Mawero 32	+	+	+			+	+	+			-	-	-			-	-	-			-	-	+		
25. Papol 42	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
26. UGK	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
27. Papol 60	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		

28. UR4	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
29. F97	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
30. Mawero 80	+	+	+			+	+	+			-	-	-			-	-	-			+	-	-		
31. Papol 278	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
32. URI	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
33. UGH	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
34. Mela Pig	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
35. Tira 22	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
36. Mela 66	+	+	+			+	+	+			-	-	+			+	+	+			+	+	+		
37. Uganda B	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
38. Mela 24	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
39. Iyolowa 125	+	+	+			+	+	+			-	-	-			-	-	-			-	-	+		
40. Tira 168	+	+	+			-	+	+			-	-	-			+	-	-			-	-	+		
41. Papol 12	+	+	+			-	+	+			-	-	-			-	+	+			-	-	+		
42. Mela 27	+	+	+			-	+	+			-	-	-			+	+	+			+	+	+		

43. Poyem 11	+	+	+			-	+	+			-	-	-			-	-	-			-	-	+		
44. Tira 4	+	+	+			-	+	+			-	-	-			-	-	-			-	-	-		
45. Mela pig 2	+	+	+			-	+	+			-	-	-			-	-	+			-	-	+		
46. UGE	+	+	+			-	+	+			-	-	-			+	+	+			+	+	+		
47. Katerema 72	+	+	+			-	+	+			-	-	-			-	-	-			-	-	-		
48. Mawero 42	+	+	+			-	+	+			-	-	-			+	+	+			+	+	+		
49. Tira 34	-	+	-			+	+	-			-	+	-			-	-	-			-	-	+		
50. H.Taka	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
51. Uganda A	+	+	+			+	+	+			-	-	+			+	+	+			+	+	+		
52. A Kiburige	+	+	+			-	+	+			-	-	-			+	+	+			+	+	+		
53. Uganda E	+	+	+			+	+	+			-	-	+			+	+	+			+	+	+		
54. Katerema 116	+	+	+			+	+	+			-	-	-			-	+	+			+	+	+		
55. Tira 43	+	+	+			-	+	+			-	+	-			-	+	-			-	-	+		
56. Tira 92	-	+	-			+	-	-			-	-	-			-	-	-			-	-	-		
57. Tira 75	+	+	+			-	+	+			-	-	-			-	-	-			+	-	-		

58. Musikia Clone B	+	+	+			-	+	+			+	+	+			-	-	-			-	-	-		
59. Iyolowa 116	+	+	+			-	+	+			-	-	-			-	-	-			-	+	-		
60. Iyolowa 153	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
61. TH 112 IM56	+	+	+			+	+	+			-	-	-			-	+	-			-	-	-		
62. Muraz 15 IM47	+	+	+			-	+	+			-	-	+			-	-	-			-	-	-		
63. Mela 12	+	+	+			-	+	+			-	-	+			-	-	-			-	+	-		
64. Mawero 31	+	+	+			+	+	+			-	+	-			+	+	+			+	+	+		
65. F73	+	+	+			+	+	+			-	+	+			+	+	+			+	+	+		
66. Tira 27	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
67. Uganda L	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
68. F48	+	+	+			+	+	+			-	+	+			+	+	+			+	+	+		
69. Tira 68	+	+	+			+	+	+			-	-	-			-	-	-			-	-	+		
70. Tira 30	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
71. Tira 29	+	+	+			-	-	-			-	-	-			-	-	-			-	+	-		
72. Papol 371	+	+	+			+	+	+			-	-	+			-	-	-			-	-	-		

73. Mawero 85	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
74. Iyolowa 147	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
75. Muraz 3 IM46	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
76. Katerema 41	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
77. UGG 88	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
78. Mela 2	+	+	+			+	+	+			-	-	+			+	+	+			+	+	+		
79. Uganda M	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
80. Rose Akinare	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
81. Uganda B	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
82. Papol 144	+	+	+			+	+	+			-	-	-			-	-	-			-	-	-		
83. Uganda C	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		
84. Sikuda 42.90	+	+	+			+	+	+			-	-	-			-	-	-			-	-	+		
85. Sikuda 4	+	+	+			-	-	-			-	-	-			-	-	-			-	-	-		
86. D. Obwang	+	+	+			+	+	+			-	-	-			+	+	+			+	+	+		

Appendix 18 Agreement between LAMP assays and the reference PCRs using control DNA samples

Table 1. Two by two table for LAMP RIME and TBR PCR

		LAMP RIME i			**LAMP RIME ii**			**LAMP RIME iii**		
		+	**-**	**Total**	**+**	**-**	**Total**	**+**	**-**	**Total**
TBR PCR	**+**	80	2	82	82	0	82	80	2	82
	-	4	0	4	4	0	4	4	0	4
	Total	84	2	86	86	0	86	84	2	86

Table 2. Two by two table for LAMP *PfrA* and TBR PCR

		LAMP *PfrA* i			**LAMP *PfrA* ii**			**LAMP *PfrA* iii**		
		+	**-**	**Total**	**+**	**-**	**Total**	**+**	**-**	**Total**
TBR PCR	**+**	66	16	82	81	1	82	80	2	82
	-	1	3	4	2	2	4	2	2	4
	Total	67	19	86	83	3	86	82	4	86

Table 3. Two by two table for LAMP *TgsGP* and *TgsGP* PCR

		LAMP *TgsGP* i			**LAMP *TgsGP* ii**			**LAMP *TgsGP* iii**		
		+	**-**	**Total**	**+**	**-**	**Total**	**+**	**-**	**Total**
***TgsGP* PCR**	**+**	8	1	9	6	3	9	8	1	9
	-	0	77	77	5	72	77	9	68	77
	Total	8	78	86	11	75	86	17	69	86

Table 4. Two by two table summarising the agreement between the three repeats of LAMP *SRA*1 and *SRA* PCR

		LAMP *SRA*1 i			**LAMP *SRA*1 ii**			**LAMP *SRA*1 iii**		
		+	-	**Total**	+	-	**Total**	+	-	**Total**
SRA **PCR**	+	33	3	36	34	2	36	34	2	36
	-	1	49	50	4	46	50	3	47	50
	Total	34	52	86	38	48	86	37	49	86

Table 5. Two by two table summarising the agreement between the three repeats of SRALAMP_a and *SRA* PCR

		SRALAMP_a i			**SRALAMP_a ii**			**SRALAMP_a iii**		
		+	-	**Total**	+	-	**Total**	+	-	**Total**
SRA **PCR**	+	34	2	36	34	2	36	34	2	36
	-	2	48	50	5	45	50	11	39	50
	Total	36	50	86	39	47	86	45	41	86

Table 6. LAMP assay agreement with reference PCR results

PCR	LAMP	κ_{max}	Prevalence index	Bias index	$\kappa_{observed}$	95% CI
TBR	RIME i	0.656	0.930	0.023	-0.032	(-0.828, 0.764)
	RIME ii	$\kappa_{observed}$	0.953	0.046	0	(-0.957, 0.957)
	RIME iii	0.656	0.930	0.023	-0.032	(-0.828, 0.764)
TBR	*PfrA* i	0.294	0.732	0.174	0.199	(-0.142, 0.540)
	PfrA ii	0.851	0.919	0.012	0.554	(0.057, 1.050)
	PfrA iii	1	0.907	0	0.476	(-0.026, 0.977)
TgsGP	*TgsGP* i	$\kappa_{observed}$	0.802	0.012	0.935	(0.808, 1.062)
	TgsGP ii	0.887	0.767	0.023	0.548	(0.250, 0.846)
	TgsGP iii	0.644	0.698	0.093	0.554	(0.295, 0.814)
SRA	*SRA*1 i	0.952	0.186	0.023	0.904	(0.812, 0.996)
	*SRA*1 ii	0.953	0.140	0.023	0.858	(0.748, 0.968)
	*SRA*1 iii	0.976	0.151	0.012	0.881	(0.780, 0.982)
SRA	SRALAMP_a i	1	0.163	0	0.904	(0.813, 0.996)
	SRALAMP_a ii	0.929	0.128	0.035	0.835	(0.717, 0.952)
	SRALAMP_a iii	0.792	0.058	0.105	0.700	(0.550-0.850)

Appendix 19. LAMP test-retest agreement using control DNA samples.

Table 1. Two by two table for comparisons of LAMP RIME repeats

		RIME ii			RIME iii		
		+	-	Total	+	-	Total
RIME i	+	84	0	84	84	0	84
	-	2	0	2	0	2	2
	Total	86	0	86	84	2	86
RIME iii	+	84	0	84			
	-	2	0	2			
	Total	86	0	86			

Table 2. Two by two table for comparisons of LAMP *PfrA* repeats

		PfrA ii			*PfrA* iii		
		+	-	Total	+	-	Total
PfrA i	+	66	1	67	65	2	67
	-	17	2	19	17	2	19
	Total	83	3	86	82	4	86
PfrA iii	+	82	0	82			
	-	1	3	4			
	Total	83	3	86			

Table 3. Two by two table for comparisons of LAMP *TgsGP* repeats

		***TgsGP* ii**			***TgsGP* iii**		
		+	-	**Total**	+	-	**Total**
***TgsGP* i**	+	6	2	8	8	0	8
	-	5	73	78	9	69	78
	Total	11	75	86	17	69	86
***TgsGP* iii**	+	8	9	17			
	-	3	66	69			
	Total	11	77	86			

Table 4. Two by two table for comparisons of LAMP *SRA*1 repeats

		***SRA*1 ii**			***SRA*1 iii**		
		+	-	**Total**	+	-	**Total**
***SRA*1 i**	+	33	1	34	33	1	34
	-	5	47	52	4	48	52
	Total	38	48	86	37	49	86
***SRA*1 iii**	+	35	2	37			
	-	3	46	49			
	Total	38	48	86			

Table 5. Two by two table for comparisons of SRALAMP_a repeats

		SRALAMP_a ii			SRALAMP_a iii		
		+	-	**Total**	+	-	**Total**
SRALAMP_a i	+	34	2	36	34	2	36
	-	5	45	50	11	39	50
	Total	39	47	86	45	41	86
SRALAMP_a iii	+	35	10	45			
	-	4	37	41			
	Total	39	47	86			

Table 6. LAMP assay test-retest agreement

LAMP assay comparisons		**κ_{max}**	**Prevalence index**	**Bias index**	**$\kappa_{observed}$**	**95% confidence interval**
RIME	i - ii	$\kappa_{observed}$	0.977	0.023	0	(-1.370, 1.370)
	i - iii	$\kappa_{observed}$	0.953	0	1	(1,1)
	ii - iii	$\kappa_{observed}$	0.977	0.023	0	(-1.370, 1.370)
PfrA	i - ii	0.226	0.744	0.186	0.129	(-0.228, 0.487)
	i - iii	0.294	0.733	0.185	0.105	(-0.250, 0.460)
	ii - iii	$\kappa_{observed}$	0.975	0.012	0.851	(0.561, 1.141)
TgsGP	i - ii	0.823	0.779	0.035	0.587	(0.294, 0.880)
	i - iii	$\kappa_{observed}$	0.709	0.105	0.588	(0.333, 0.843)
	ii - iii	0.746	0.674	0.070	0.493	(0.226, 0.759)
*SRA*1	i - ii	0.905	0.163	0.047	0.857	(0.747, 0.962)
	i - iii	0.928	0.174	0.035	0.880	(0.778, 0.982)
	ii - iii	0.953	0.128	0.012	0.882	(0.781, 0.982)
SRALAMP_a	i - ii	0.929	0.128	0.035	0.835	(0.717, 0.952)
	i - iii	0.792	0.058	0.104	0.700	(0.550, 0.850)
	ii - iii	0.861	0.023	0.070	0.676	(0.520, 0.831)

Appendix 20. LAMP and PCR test-retest agreement using DNA eluted from cattle blood samples on Whatman FTA cards

Table 1. Two by two table for comparisons of LAMP RIME repeats

		RIME ii			RIME iii		
		+	-	Total	+	-	Total
RIME i	+	9	8	17	7	10	17
	-	5	26	31	12	19	31
	Total	14	34	48	19	29	48
RIME iii	+	8	11	19			
	-	6	23	29			
	Total	14	34	48			

Table 2. Two by two table for comparisons of TBR PCR repeats

		TBR PCR ii			*TBR PCR* iii		
		+	-	Total	+	-	Total
TBR PCR i	+	21	2	23	23	0	23
	-	1	31	25	1	24	25
	Total	22	34	48	24	24	48
TBR PCR iii	+	21	5	24			
	-	2	29	24			
	Total	22	34	48			

Table 3. LAMP and PCR test-retest agreement using DNA prepared from cattle blood samples

LAMP assay comparisons		**κ_{max}**	**Prevalence index**	**Bias index**	**$\kappa_{observed}$**	**95% confidence interval**
TBR PCR	i - ii	0.958	0.063	0.021	0.875	(0.737, 1.012)
	i - iii	$\kappa_{observed}$	0.021	0.021	0.958	(0.878, 1.039)
	ii - iii	0.840	0.042	0.042	0.833	(0.677, 0.990)
RIME	i - ii	0.858	0.354	0.063	0.383	(0.097, 0.670)
	i - iii	0.911	0.25	0.042	0.024	(-0.276, 0.324)
	ii - iii	0.772	0.313	0.104	0.224	(-0.072, 0.521)

Appendix 21. LAMP and PCR test-retest agreement using DNA eluted from human blood samples on Whatman FTA cards

Table 1. Two by two table for comparisons of LAMP RIME repeats

		RIME ii			RIME iii		
		+	-	Total	+	-	Total
RIME i	+	41	3	44	43	1	44
	-	7	1	8	6	2	8
	Total	48	4	52	49	3	52
RIME iii	+	46	3	49			
	-	2	1	3			
	Total	48	4	52			

Table 2. Two by two table for comparisons of TBR PCR repeats

		TBR PCR ii			*TBR PCR* iii		
		+	-	Total	+	-	Total
TBR PCR i	+	52	0	52	52	0	52
	-	0	0	0	0	0	0
	Total	52	0	52	52	0	52
TBR PCR iii	+	52	0	52			
	-	0	0	0			
	Total	52	0	52			

Table 3. Three by three table for comparisons of *SRA* PCR repeats

		***SRA* PCR ii**				***SRA* PCR iii**			
		+	-	**Insufficient DNA**	**Total**	+	-	**Insufficient DNA**	**Total**
***SRA* PCR i**	+	30	5	0	35	30	4	0	34
	-	4	2	2	8	3	3	2	8
	Insufficient DNA	3	2	4	9	1	2	6	9
	Total	37	9	6	52	34	9	8	51
***SRA* PCR iii**	+	29	5	0	34				
	-	6	2	1	9				
	Insufficient DNA	2	1	5	8				
	Total	37	8	6	51				

Table 4. Two by two table for comparisons of LAMP *SRA*1 repeats

		***SRA*1 ii**			***SRA*1 iii**		
		+	-	**Total**	+	-	**Total**
***SRA*1 i**	+	41	1	42	39	3	42
	-	0	10	10	3	7	10
	Total	41	11	52	39	13	52
***SRA*1 iii**	+	36	3	39			
	-	5	8	13			
	Total	41	11	52			

Table 5. Two by two table for comparisons of SRALAMP_a repeats

		SRALAMP_a ii			SRALAMP_a iii		
		+	-	**Total**	+	-	**Total**
SRALAMP_a i	+	8	12	20	7	13	20
	-	2	30	32	11	21	32
	Total	10	42	52	18	34	52
SRALAMP_a iii	+	4	14	18			
	-	6	28	34			
	Total	10	42	52			

Table 6. LAMP and PCR test-retest agreement using DNA prepared from human blood samples

LAMP assay comparisons		κ_{max}	Prevalence index	Bias index	$\kappa_{observed}$	95% confidence interval
TBR PCR	i - ii		1	0		
	i - iii		1	0		
	ii - iii		1	0		
RIME	i - ii	0.629	0.769	0.077	0.071	(-0.446, 0.589)
	i - iii	0.504	0.788	0.096	0.305	(-0.173, 0.784)
	ii - iii	0.847	0.865	0.019	0.235	(-0.402, 0.873)
SRA PCR	i - ii				0.352	(0.087, 0.616)
	i - iii				0.530	(0.297, 0.762)
	ii - iii				0.374	(0.109, 0.640)
*SRA*1	i - ii	0.940	0.596	0.019	0.463	(0.144, 0.782)
	i - iii	0.833	0.558	0.058	0.500	(0.203, 0.797)
	ii - iii	0.892	0.538	0.038	0.568	(0.292, 0.843)
SRALAMP_a	i - ii	0.552	0.423	0.192	0.372	(0.091, 0.653)
	i - iii	0.917	0.269	0.038	0.006	(-0.285, 0.298)
	ii - iii	0.620	0.462	0.154	0.051	(-0.275, 0.377)

Appendix 22. LAMP and PCR test-retest agreement using pooled data from cattle and human sample sets

Table 1. Two by two table for comparisons of LAMP RIME repeats

		RIME ii			RIME iii		
		+	-	Total	+	-	Total
RIME i	+	50	11	61	50	11	61
	-	12	27	39	18	21	39
	Total	62	38	100	68	32	100
RIME iii	+	54	14	68			
	-	8	24	32			
	Total	62	38	100			

Table 2. Two by two table for comparisons of TBR PCR repeats

		TBR PCR ii			*TBR PCR* iii		
		+	-	Total	+	-	Total
TBR PCR i	+	73	2	75	75	0	75
	-	1	24	25	1	24	25
	Total	74	26	100	76	24	100
TBR PCR iii	+	73	3	76			
	-	1	23	24			
	Total	74	26	100			

Table 3. Three by three table for comparisons of *SRA* PCR repeats

		***SRA* PCR ii**				***SRA* PCR iii**			
		+	**-**	**Insufficient DNA**	**Total**	**+**	**-**	**Insufficient DNA**	**Total**
***SRA* PCR i**	**+**	31	5	0	36	30	5	0	35
	-	5	3	7	15	3	5	7	15
	Insufficient DNA	3	2	44	49	1	6	42	49
	Total	39	10	51	100	34	16	49	99
***SRA* PCR iii**	**+**	29	5	0	34				
	-	7	2	7	16				
	Insufficient DNA	3	1	44	49				
	Total	39	9	51	99				

Table 4. Two by two table for comparisons of LAMP *SRA*1 repeats

		***SRA*1 ii**			***SRA*1 iii**		
		+	**-**	**Total**	**+**	**-**	**Total**
***SRA*1 i**	**+**	39	11	50	39	11	50
	-	5	45	50	10	40	50
	Total	44	56	100	49	51	100
***SRA*1 iii**	**+**	37	12	49			
	-	7	44	51			
	Total	44	56	100			

Table 5. Two by two table for comparisons of SRALAMP_a repeats

		SRALAMP_a ii			SRALAMP_a iii		
		+	-	**Total**	+	-	**Total**
SRALAMP_a i	+	9	13	22	8	14	22
	-	3	75	78	12	66	78
	Total	12	88	100	20	80	100
SRALAMP_a iii	+	5	15	20			
	-	7	73	80			
	Total	12	88	100			

Table 6. LAMP and PCR test – retest agreement pooling all data from cattle and human samples

LAMP assay comparisons		**κ_{max}**	**Prevalence index**	**Bias index**	**$\kappa_{observed}$**	**95% confidence interval**
TBR PCR	i - ii	0.974	0.490	0.010	0.921	(0.833, 1.009)
	i - iii	$\kappa_{observed}$	0.510	0.010	0.973	(0.920, 1.026)
	ii - iii	0.947	0.500	0.020	0.893	(0.791, 0.996)
RIME	i - ii	0.979	0.230	0.010	0.514	(0.340, 0.689)
	i - iii	0.848	0.290	0.070	0.370	(0.177, 0.563)
	ii - iii	0.869	0.300	0.060	0.518	(0.341, 0.696)
SRA PCR	i - ii				0.630	(0.494, 0.767)
	i - iii				0.635	(0.501, 0.770)
	ii - iii				0.605	(0.463, 0.746)
*SRA*1	i - ii	0.880	0.060	0.060	0.680	(0.536, 0.824)
	i - iii	0.980	0.010	0.010	0.580	(0.420, 0.740)
	ii - iii	0.900	0.070	0.050	0.619	(0.465, 0.773)
SRALAMP_a	i - ii	0.652	0.660	0.100	0.443	(0.193, 0.693)
	i - iii	0.940	0.580	0.020	0.217	(-0.042, 0.476)
	ii - iii	0.191	0.680	0.080	0.191	(-0.107, 0.490)

Appendix 23. LAMP assays with calcein and $MnCl_2$

Table 1. LAMP *PfrA* with Tween

	Replicate 1			Replicate 2			Replicate 3		
Dilution	Colour	Fluor.	Gel	Colour	Fluor.	Gel	Colour	Fluor.	Gel
1×10^{-1}	Green?	+	+	Green/orange	+?	+	Orange	+?	+
1×10^{-2}	Green?	+	+	Green	-	+	Orange	+?	+
1×10^{-3}	Green?	+	+	Green	-	-	Orange	+?	-
1×10^{-4}	Orange	-	-	Green	-	-	Orange	+?	-
1×10^{-5}	Orange	-	-	Orange	-	-	Orange	+?	-
1×10^{-6}	Orange	-	-	Orange	-	-	Orange	+?	-
1×10^{-7}	Orange	-	-	Orange	-	-	Orange	+?	-

Table 2. LAMP *SRA*1 with Tween

	Replicate 1			Replicate 2			Replicate 3		
Dilution	Colour	Fluor.	Gel	Colour	Fluor.	Gel	Colour	Fluor.	Gel
1×10^{-1}	Green	++	+	Orange	++	-	Not done		
1×10^{-2}	Green	++	+	Orange	+	-	Not done		
1×10^{-3}	Green/Orange	++	+	Orange	+	-	Not done		
1×10^{-4}	Orange	-	-	Orange	+	-	Not done		
1×10^{-5}	Orange	-	+	Orange	+	-	Not done		
1×10^{-6}	Orange	-	-	Orange	-	-	Not done		
1×10^{-7}	Orange	-	-	Orange	+	-	Not done		

Table 3. SRALAMP_a with Triton

	Replicate 1			Replicate 2			Replicate 3		
Dilution	Colour	Fluor.	Gel	Colour	Fluor.	Gel	Colour	Fluor.	Gel
1×10^{-1}	Green	+	+	Orange	-	-	Orange	+	+
1×10^{-2}	Orange	-	-	Orange	-	-	Orange	+	+
1×10^{-3}	Orange	-	-	Orange	-	-	Orange	-	+
1×10^{-4}	Orange	-	-	Orange	-	-	Orange	-	+
1×10^{-5}	Orange	-	-	Orange	-	-	Orange	-	-
1×10^{-6}	Orange	-	-	Orange	-	-	Orange	-	-
1×10^{-7}	Orange	-	-	Orange	-	-	Orange	-	-

Table 4. SRALAMP_a with Tween

	Replicate 1			Replicate 2			Replicate 3		
Dilution	Colour	Fluor.	Gel	Colour	Fluor.	Gel	Colour	Fluor.	Gel
1×10^{-1}	Green	+	+	Green	++	+	Green	+	+
1×10^{-2}	Green	+	+	Green	++	+	Green	+	-
1×10^{-3}	Orange	-	-	Green/Orange	++	+	Green	+	Weak +
1×10^{-4}	Orange	-	-	Orange	-	-	Green	+	-
1×10^{-5}	Orange	-	-	Orange	-	+	Green	+	-
1×10^{-6}	Orange	-	-	Orange	-	-	Green	+	+
1×10^{-7}	Orange	-	-	Orange	-	-	Green	+	-

Appendix 24. Turbidity verus gel electrophoresis for LAMP *PfrA*, SRALAMP_a and LAMP *TgsGP* for the total set of control DNA samples

LAMP *PfrA*

Table 1. Two by two tables comparing turbidity with UV illumination after gel electrophoresis for reading the results of LAMP *PfrA* reactions for three separate screens of the sample set

		(i)			(ii)			(iii)		
		Turbidity			Turbidity			Turbidity		
		+	-	Total	+	-	Total	+	-	Total
Gel	+	59	8	67	81	2	83	77	5	82
	-	0	19	19	1	2	3	0	4	4
	Total	59	27	86	82	4	86	77	9	86

Table 2. Two by two table comparing turbidity with UV illumination after gel electrophoresis for reading the results of LAMP *PfrA* reactions for three screens of the sample set combined

		Turbidity		
		+	-	Total
Gel	+	217	15	232
	-	1	25	26
	Total	218	40	258

SRALAMP_a

Table 3. Two by two tables comparing turbidity with UV illumination after gel electrophoresis for reading the results of SRALAMP_a reactions for three separate screens of the sample set

		(i)			**(ii)**			**(iii)**		
		Turbidity			**Turbidity**			**Turbidity**		
		+	-	**Total**	+	-	**Total**	+	-	**Total**
Gel	+	16	20	36	37	2	39	42	3	45
	-	0	47	47	0	44	44	0	38	38
	Total	16	67	83	37	46	83	42	41	83

Table 4. Two by two table comparing turbidity with UV illumination after gel electrophoresis for reading the results of SRALAMP_a reactions for three screens of the sample set combined

		Turbidity		
		+	-	**Total**
Gel	+	95	25	120
	-	0	129	129
	Total	95	154	249

LAMP *TgsGP*

Table 5. Two by two tables comparing turbidity with UV illumination after gel electrophoresis for reading the results of LAMP *TgsGP* reactions for three separate screens of the sample set

		(i)			**(ii)**			**(iii)**		
		Turbidity			**Turbidity**			**Turbidity**		
		+	**-**	**Total**	+	**-**	**Total**	+	**-**	**Total**
Gel	+	5	3	8	7	4	11	12	3	15
	-	0	75	75	0	72	72	0	68	68
	Total	5	78	83	7	76	83	12	71	83

Table 6. Two by two table comparing turbidity with UV illumination after gel electrophoresis for reading the results of LAMP *TgsGP* reactions for three screens of the sample set combined

		Turbidity		
		+	**-**	**Total**
Gel	+	24	10	34
	-	0	215	215
	Total	24	225	249